

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property
Organization
International Bureau



(43) International Publication Date
9 December 2004 (09.12.2004)

PCT

(10) International Publication Number
WO 2004/106356 A1

(51) International Patent Classification⁷: **C07H 19/06**,
19/10, 19/16, 19/20, 21/00, C12N 15/11, A61K 31/7064,
31/7076, 31/712, A61P 31/12, 35/00

(21) International Application Number:
PCT/DK2004/000372

(22) International Filing Date: 27 May 2004 (27.05.2004)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
PA 2003 00804 27 May 2003 (27.05.2003) DK

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(81) Designated States (unless otherwise indicated, for every
kind of national protection available): AE, AG, AL, AM,
AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN,
CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI,
GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE,
KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD,
MG, MK, MN, MW, MX, MZ, NA, NI, NO, NZ, OM, PG,
PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM,
TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM,
ZW.

(84) Designated States (unless otherwise indicated, for every
kind of regional protection available): ARIPO (BW, GH,
GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM,
ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM),
European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI,
FR, GB, GR, HU, IE, IT, LU, MC, NL, PL, PT, RO, SE, SI,
SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ,
GW, ML, MR, NE, SN, TD, TG).

Published:

- with international search report
- with amended claims

For two-letter codes and other abbreviations, refer to the "Guid-
ance Notes on Codes and Abbreviations" appearing at the begin-
ning of each regular issue of the PCT Gazette.

(54) Title: FUNCTIONALIZED NUCLEOTIDE DERIVATIVES

(57) Abstract: A nucleotide derivative is disclosed, which in its 4' and/or 5' position on the sugar moiety is substituted with a group comprising a non-aromatic cyclid group comprising at least one nitrogen atom, said cyclid group optionally being substituted. The nucleotide monomer induces increased thermal stability of RNA:DNA, RNA:RNA or DNA:DNA duplexes, if said RNA and/or DNA strand comprises at least one said monomer. The nucleotides and oligonucleotides comprising at least one of said nucleotides can be used in therapy (such as antisense or antigene therapy) and in methods for synthesising polynucleotides (e.g. as a primer). The nucleotide derivative may also be used in a medicament. Further, the use of the nucleotide derivative for the preparation of a medicament for the treatment of diseases or disorders is also disclosed.

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FUNCTIONALIZED NUCLEOTIDE DERIVATIVES**FIELD OF THE INVENTION**

5 Derivatives of oligonucleotides have been prepared in order to augment their therapeutic utility in the field of antisense and antigene therapy. Conjugation at the 4' or 5' position of a nucleoside within an oligonucleotide has led to new chemical entities with relevant binding affinity to complementary oligonucleotides

10 BACKGROUND

Manoharan has described the preparation of various conjugated oligonucleotides (ONs) and their therapeutic potential as antisense ONs.¹

15 ONs conjugated with alkylamines at the phosphate,² base³⁻⁶ and sugar⁷⁻¹⁰ moieties have been prepared and their binding towards complementary ONs studied.

We and others have during the recent years studied the effect of incorporating a number of C-hydroxymethylated monomers into ONs, including functionalized 5'-C-alkyl¹¹⁻¹⁵ and 4'-C-alkyl nucleotides^{8-10,16-19} (see Figure 1 for selected structures of monomers, *e.g.*,
20 monomers **A** and **B**).

In order to improve the therapeutic and diagnostic potential of antisense ONs, there is a need for oligonucleotides having a strong binding affinity towards a target poly- or oligonucleotide.

25

SUMMARY OF THE INVENTION

It is an objective of this invention to provide nucleotides and oligonucleotides with strong or preferably improved binding affinity compared to natural occurring nucleotides and oligonucleotides containing such nucleotides.

30

Towards this end, the invention relates to a nucleotide derivative which in its 4' and/or 5' position on the sugar moiety is substituted with a group comprising a non-aromatic cyclic group comprising at least one nitrogen atom, said cyclic group optionally being substituted. Compounds of the formula I anticipated by the present invention.

35

It is a further object of the present invention to provide nucleotides and oligonucleotides which have a strong binding affinity under conditions where pH is low or reduced relative to standard conditions and/or the concentration of salt or ions (*e.g.* sodium) is low or reduced.

40

It is a further objective of the invention to provide a nucleotide monomer which induces increased thermal stability of a RNA:DNA, RNA:RNA or DNA:DNA duplex, if said RNA and/or DNA strand comprises at least one said monomer. Specifically, it is an objective to provide nucleotide monomers that display a clear thermal preference of hybridizing

45 towards DNA rather than RNA.

It is also an objective of the present invention to provide nucleotides and oligonucleotides, which can be used in therapy (such as antisense therapy); in diagnostics (e.g. as a primer or probe, eg. when labeled); in methods for synthesising polynucleotides (e.g. as a
5 primer).

It is also an objective of the present invention to provide nucleotides and oligonucleotides, which can be used for conjugation to, e.g., peptides or other groups, thereby leading to improved effect as antisense agent or novel diagnostic probes.

10

It is a further object of the invention to provide a pharmaceutical composition comprising at least one nucleotide and/or oligonucleotide and/or polynucleotide according to any of the preceding claims, and a pharmaceutically acceptable carrier.

15 A related object of the invention is directed to a nucleotide derivative as described herein for use as a medicament.

It is also an objective of the present invention to provide oligonucleotides that are able to bind to double stranded DNA. Thus, a DNA selective binding ability, i.e. the ability to bind
20 to complementary DNA and not to complementary RNA, allows the combined use of an oligonucleotide of this invention together with an RNA of the complementary sequence for simultaneous targeting of both strands of double stranded DNA. Instead of RNA, an RNA analogue like 2'-O-Me-RNA or other 2'-O-alkyl-RNA derivatives or LNA or the like can be applied in combination with an oligonucleotide of the invention.

25

It has surprisingly turned out that the above objectives are met by the novel nucleotides of the invention, and by the novel oligonucleotides comprising at least one nucleotide.

The nucleotides of the invention are characterized in that they contain a nitrogen
30 containing ring structure in the sugar moiety, preferably in the 4' or 5' position. It is assumed that a nitrogen atom ring in the ring structure is protonated under physiological conditions, thus increasing the binding affinity towards the negatively charged target strands.

35 A further object of the invention relates to the use of a nucleotide of the invention for the preparation of a medicament for the treatment of a disease or disorder selected from cancer; diseases caused by viral infections, such as AIDS; influenza, angiogenesis; atherosclerosis, psoriasis, diabetic retinopathy, rheumatoid arthritis, asthma, warts, allergic dermatitis and Kaposi sarcoma.

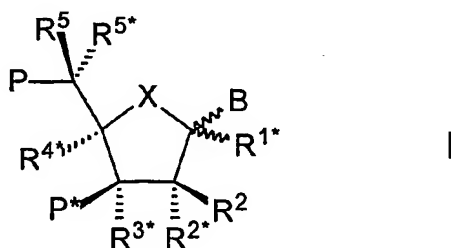
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DETAILED DISCLOSURE OF THE INVENTION

According to the above finding, the present invention relates to novel nucleotide derivatives which in the 4' and/or 5' position on the sugar moiety is substituted with a group comprising a non-aromatic cyclic group comprising at least one nitrogen atom, said

5 cyclic group optionally being substituted.

In a presently preferred embodiment, the nucleoside derivatives of the invention has the structure I as depicted in claim 2 (in any configuration):



10

wherein X is selected from -O-, -S-, -N(R⁶)-, -C(R⁶R^{6*})-;

B is selected from hydrogen, hydroxy, optionally substituted C₁₋₄-alkoxy, optionally substituted C₁₋₄-alkyl, optionally substituted C₁₋₄-acyloxy, nucleobases (preferably a base selected from the group consisting of Adenine, Guanine, Cytosine, Uracil, Thymine and derivatives thereof);

15

P designates the radical position for an internucleoside linkage to a succeeding monomer, or a 5'-terminal group such as OH or a OH protection group ((OB, B=blocking group) eg. OCEPA, OTs,);

20

P* designates an internucleoside linkage to a preceding monomer, or a 3'-terminal group such as OH or a OH protection group (eg. OCEPA, OTs);

25

each of the substituents R^{1*}, R², R^{2*}, R^{3*}, R^{4*}, R⁵, R^{5*}, R⁶, and R^{6*} is independently selected, preferably from hydrogen, alkyl, alkenyl, alkynyl, hydroxy, alkoxy, alkenyloxy, mercapto, alk(en)ylthio, carboxy, alkoxycarbonyl, alkylcarbonyl, formyl, aryl, aralkyl, aryl-oxy-carbonyl, aryloxy, arylthio, aralkoxy, arylcarbonyl, heterocyclyl, heterocyclyl-alkyl, heterocycloxy-carbonyl, heterocyclyl-oxy, heterocyclyl-carbonyl, amino, mono- and di(alkyl)amino, carbamoyl, mono- or di(alkyl)-amino-carbonyl, amino-alkyl-aminocarbonyl, mono- or di(alkyl)amino-alkylaminocarbonyl, alkyl-carbonylamino, carbamido, alkanoyloxy, sulphonyloxy, alkylsulphonyloxy, nitro, azido, sulphonyl, alkylthio, halogen;

30

any of said above optionally being substituted (eg by replacing one or more (such as 2, 3, 4, 5, 6, 7, or more hydrogen atoms) with one or more groups known to the skilled person, preferably selected from:

35

C₁-C₆ alkyl, C₁-C₆ alkoxy, aralkyl, aryl, heterocyclyl, acyl, halogen, nitro, hydroxy, amino, CN, N₃, CF₃, NR₂, OH, OR, SH, SR, OR, COOH, COOR, SO₃-R, where R is selected from the group consisting of H, alkyl, aralkyl, aryl, acyl, CF₃-CO;

- 5 and/or where two geminal substituents together may designate oxo, thloxo, imino, or optionally substituted methylene, the substituents defined above;

provided that at least one of the substituents R^{4*} and R^{5*} contains a nitrogen containing non-aromatic heterocyclic ring;

10

and salts, such as basic salts and acid addition salts thereof.

It should be understood that hydrocarbon radicals or groups, such as alkyl, alkenyl, and alkynyl, or when such groups are a part of a combination with another radical or radicals,

- 15 eg. aralkyl, they are saturated or unsaturated C₁-C₁₂, preferably C₁-C₆.

It is preferred that R^{1*}, R², R^{3*}, and R⁵ independently are selected from the group consisting of hydrogen, alkyl, substituted alkyl, aralkyl, substituted aralkyl, aryl, and substituted aryl, the substituents being defined as above; that R^{2*} is selected from the
 20 group consisting of hydrogen, hydroxy, alkyl, substituted alkyl, aralkyl, substituted aralkyl, aryl, and substituted aryl, the substituents being defined as above; and that the nitrogen containing non-aromatic heterocyclic ring (contained in R^{4*} and/or R^{5*}) is linked to the sugar moiety of the nucleotide by a group selected from: C₁-C₁₂ alkylene (eg. methylene, ethylene); C₁-C₁₂ alkylene wherein one or more carbon atoms are replaced with a
 25 heteroatom (eg. oxymethylene, methyleneoxy, methylenelmino, thiomethylene, methylenethio); C=O (carbonyl); a heteroatom (eg. oxy or thio); N-R⁶ (R⁶ defined as above); or a combination of two or more of these groups.

- In a preferred embodiment, the non-aromatic nitrogen-containing heterocyclic ring in R^{4*}
 30 and/or R^{5*} is selected from a ring having 5, 6 or 7 ring members, said ring members being at least one nitrogen, and the rest of the ring members are independently selected from carbon, oxygen, sulphur and nitrogen; said ring containing no or one double bond; and said ring optionally being substituted with a group preferably selected from: C₁-C₆ alkyl, C₁-C₆ alkoxy, aralkyl, aryl, acyl, halogen, nitro, hydroxy, amino, CN, N₃, CF₃, NR₂, OH, OR,
 35 SH, SR, OR, COOH, COOR, SO₃-R, where R is selected from the group consisting of H, alkyl, aralkyl, aryl, acyl, CF₃-CO. Most preferred is a ring selected from piperazine, piperidine, pyrrolidine or morpholine; any of which is optionally substituted with a substituent R⁶ as defined above; and being coupled to the linker via a ring carbon or nitrogen atom. Examples are 4'-C-(4-methylpiperazino)methyl) - or 4'-C-
 40 (piperazino)methyl)-derivatives.

- In a further preferred embodiment, the non-aromatic nitrogen-containing heterocyclic ring in R^{4*} and/or R^{5*} having 5, 6 or 7 ring members, said ring members being at least one
 45 oxygen, sulphur and nitrogen; said ring containing no or one double bond; said ring is

optionally substituted with a group selected from aralkyl and acyl. Most preferred is a ring selected from piperazine, piperidine, pyrrolidine or morpholine; optionally substituted with aralkyl or acyl; and being coupled to the linker via a ring carbon or nitrogen atom.

- 5 In a particularly preferred embodiment, the non-aromatic nitrogen-containing heterocyclic ring in R^{4*} and/or R^{5*} is a ring selected from piperazine, piperidine, pyrrolidine or morpholine; optionally substituted with R⁷-alkyl, R⁷-alkyl-carbonyl, or R⁷-alkyl-thiocarbonyl, R⁷ being an aromatic or non-aromatic, cyclic or heterocyclic ring-system with from 6 to 24 atoms, such as indoliziny, indolyl, isoindolyl, indoliny, benzofuranyl, benzothiophenyl, indazolyl, benzimidazolyl, benzthiazolyl, puriny, quinoliziny, quinoliny, isoquinoliny, cinnoliny, phtalaziny, quinazoliny, quinoxaliny, naphthyridiny, pteridiny, chromanyl, isochromanyl, carbazolyl, acridiny, phenaziny, phenothiaziny, phenoxaziny, thianthrenyl, indenyl, naphthalenyl, anthracenyl, acenaphthylenyl, fluorenyl, phenathrenyl, biphenylenyl, phenalenyl, tetracenyl, pyrenyl, triphenylenyl, chrysenyl, aceanthrylenyl, acephenanthrylenyl, fluorantheny, pleiadenyl, perylenyl, chromenyl, isochromenyl, xanthylenyl, phenanthridiny, benztriazolyl, peridimidiny, phenanthroliny, and oxanthrenyl; and being coupled to the linker via a ring carbon or nitrogen atom. In one preferred embodiment, said ring is substituted with optionally substituted pyren-1-yl-carbonyl or pyren-1-yl-butanoyl.

20

In an alternate embodiment, R⁷ is not pyrenyl.

- Another embodiment of the invention relates to oligonucleotides containing at least one nucleoside derivatives, such as 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 20, 30, 40, 25 100 or more derivatives of the invention. It is preferred that an oligonucleotide of the invention contains has a length of 5-50 nucleotide units, preferably 10-30 units, most preferably 12-20 units. Optionally, the oligonucleotide is labelled.

- In a further embodiment, the invention relates to polynucleotides which contains at least 30 one nucleotide or oligonucleotide according to the invention.

- In such a polynucleotide or oligonucleotide according to the invention, any internucleoside linkage (the divalent linker group that forms the covalent linking of two adjacent nucleosides, between the 3' carbon atom on the first nucleoside and the 5' carbon atom on the second nucleoside (said nucleosides optionally being 3',5' dideoxy)) is selected 35 independently from linkages consisting of 2 to 4, preferably 3, groups/atoms selected from -CH₂-, -O-, -S-, -NR^H-, >C=O, >C=NR^H, >C=S, -Si(R'')₂-, -SO-, -S(O)₂-, -P(O)₂-, -P(O,S)-, -P(S)₂-, -PO(R'')-, -PO(OCH₃)-, and -PO(NHR^H)-, where R^H is selected from hydrogen and C₁₋₄-alkyl, and R'' is selected from C₁₋₆-alkyl and phenyl. It is understood that the groups are combined freely to form the linkage. It is presently preferred that the internucleoside linkage is selected from the group consisting of -CH₂-CH₂-CH₂-, -CH₂-CO-CH₂-, -CH₂-CHOH-CH₂-, -O-CH₂-O-, -O-CH₂-CH₂-, -O-CH₂-CH=, -CH₂-CH₂-O-, -NR^H-CH₂-CH₂-, -CH₂-CH₂-NR^H-, -CH₂-NR^H-CH₂-, -O-CH₂-CH₂-NR^H-, -NR^H-CO-O-, -NR^H-CO-NR^H-, -NR^H-CS-NR^H-, -NR^H-C(=NR^H)-NR^H-, -NR^H-CO-CH₂-NR^H-, -O-CO-O-, -O-CO-CH₂-O-, -O-CH₂-CO-O-, -CH₂-CO-NR^H-, -O-CO-NR^H-, -NR^H-CO-CH₂-, -O-CH₂-CO-NR^H-, -O-CH₂-CH₂-NR^H-, -CH=N-O-, 45

$-\text{CH}_2-\text{NR}^{\text{H}}-\text{O}-$, $-\text{CH}_2-\text{O}-\text{N}=\text{}$, $-\text{CH}_2-\text{O}-\text{NR}^{\text{H}}-$, $-\text{CO}-\text{NR}^{\text{H}}-\text{CH}_2-$, $-\text{CH}_2-\text{NR}^{\text{H}}-\text{O}-$, $-\text{CH}_2-\text{NR}^{\text{H}}-\text{CO}-$,
 $-\text{O}-\text{NR}^{\text{H}}-\text{CH}_2-$, $-\text{O}-\text{NR}^{\text{H}}-$, $-\text{O}-\text{CH}_2-\text{S}-$, $-\text{S}-\text{CH}_2-\text{O}-$, $-\text{CH}_2-\text{CH}_2-\text{S}-$, $-\text{O}-\text{CH}_2-\text{CH}_2-\text{S}-$, $-\text{S}-\text{CH}_2-\text{CH}=\text{}$,
 $-\text{S}-\text{CH}_2-\text{CH}_2-$, $-\text{S}-\text{CH}_2-\text{CH}_2-\text{O}-$, $-\text{S}-\text{CH}_2-\text{CH}_2-\text{S}-$, $-\text{CH}_2-\text{S}-\text{CH}_2-$, $-\text{CH}_2-\text{SO}-\text{CH}_2-$, $-\text{CH}_2-\text{SO}_2-\text{CH}_2-$,
 $-\text{O}-\text{SO}-\text{O}-$, $-\text{O}-\text{S}(\text{O})_2-\text{O}-$, $-\text{O}-\text{S}(\text{O})_2-\text{CH}_2-$, $-\text{O}-\text{S}(\text{O})_2-\text{NR}^{\text{H}}-$, $-\text{NR}^{\text{H}}-\text{S}(\text{O})_2-\text{CH}_2-$, $-\text{O}-\text{S}(\text{O})_2-\text{CH}_2-$,
5 $-\text{O}-\text{P}(\text{O})_2-\text{O}-$, $-\text{O}-\text{P}(\text{O},\text{S})-\text{O}-$, $-\text{O}-\text{P}(\text{S})_2-\text{O}-$, $-\text{S}-\text{P}(\text{O})_2-\text{O}-$, $-\text{S}-\text{P}(\text{O},\text{S})-\text{O}-$, $-\text{S}-\text{P}(\text{S})_2-\text{O}-$, $-\text{O}-\text{P}(\text{O})_2-$
 $-\text{S}-$, $-\text{O}-\text{P}(\text{O},\text{S})-\text{S}-$, $-\text{O}-\text{P}(\text{S})_2-\text{S}-$, $-\text{S}-\text{P}(\text{O})_2-\text{S}-$, $-\text{S}-\text{P}(\text{O},\text{S})-\text{S}-$, $-\text{S}-\text{P}(\text{S})_2-\text{S}-$, $-\text{O}-\text{PO}(\text{R}'')-\text{O}-$, $-\text{O}-$
 $\text{PO}(\text{OCH}_3)-\text{O}-$, $-\text{O}-\text{PO}(\text{BH}_3)-\text{O}-$, $-\text{O}-\text{P}(\text{O})_2-\text{NR}^{\text{H}}-$, $-\text{NR}^{\text{H}}-\text{P}(\text{O})_2-\text{O}-$, $-\text{O}-\text{P}(\text{O},\text{NR}^{\text{H}})-\text{O}-$, and $-\text{O}-$
 $\text{Si}(\text{R}'')_2-\text{O}-$, RH and R'' has the same meaning as R_6 above. Most preferred are
Internucleoside linkages selected from $-\text{CH}_2-\text{CO}-\text{NR}^{\text{H}}-$, $-\text{CH}_2-\text{NR}^{\text{H}}-\text{O}-$, $-\text{S}-\text{CH}_2-\text{O}-$, $-\text{O}-\text{P}(\text{O})_2-$
10 $-\text{O}-$, $-\text{O}-\text{P}(\text{O},\text{S})-\text{O}-$, $-\text{O}-\text{P}(\text{S})_2-\text{O}-$, $-\text{NR}^{\text{H}}-\text{P}(\text{O})_2-\text{O}-$, $-\text{O}-\text{P}(\text{O},\text{NR}^{\text{H}})-\text{O}-$, $-\text{O}-\text{PO}(\text{R}'')-\text{O}-$, and
 $-\text{O}-\text{PO}(\text{CH}_3)-\text{O}-$, where R^{H} is selected from hydrogen and C_{1-4} -alkyl, and R'' is selected from
 C_{1-6} -alkyl and phenyl.

In a further embodiment of the present invention, the oligonucleotide or polynucleotide
15 according to the invention has enzymatic activity (e.g. being a DNase), or is able to
bind to a polypeptide (such as an enzyme) or to a polynucleotide (such as RNA (eg siRNA)
or DNA).

In a still further embodiment, the invention relates to a composition, e.g. a pharmaceutical
20 composition, comprising at least one nucleotide and/or oligonucleotide and/or
polynucleotide according to the invention. A pharmaceutical composition comprises
preferably a pharmaceutically acceptable carrier, and at least one nucleotide and/or
oligonucleotide and/or polynucleotide according to the invention, optionally in form of a
salt.

25 In an embodiment, the oligonucleotides of the invention are adapted for use in antisense
therapy. Preferably, such ONs recruits RNase H for degradation of targeted RNA (mRNA)

In an embodiment, the oligonucleotides of the invention are adapted for use in antigene
30 therapy. Preferably, such oligonucleotides are used for targeting double stranded DNA by
its combined use together with an RNA (or RNA analogue or LNA or the like) of the
complementary sequence for simultaneous targeting of both strands of double stranded
DNA. Alternatively, targeting of double stranded DNA by triple helix formation can be
envisioned.

35 In an embodiment, the oligonucleotides of the invention are adapted for use as aptamers,
i.e. molecules able to interact with protein receptors or other molecular structures using
other modes of interaction than Watson-Crick base pairing. Such aptamers may be
generated from single stranded, double stranded or multi stranded oligonucleotides of the
40 invention.

The invention also concerns a pharmaceutical composition comprising a pharmaceutically active modified oligonucleotide or a pharmaceutically active monomer as defined above in combination with a pharmaceutically acceptable carrier.

- 5 Such compositions may be in a form adapted to oral, parenteral (Intravenous, intraperitoneal), intramuscular, rectal, intranasal, dermal, vaginal, buccal, ocularly, or pulmonary administration, preferably in a form adapted to oral administration, and such compositions may be prepared in a manner well-known to the person skilled in the art, e.g. as generally described in "Remington's Pharmaceutical Sciences", 17. Ed. Alfonso R. Gennaro (Ed.), Mark Publishing Company, Easton, PA, U.S.A., 1985 and more recent
10 editions and in the monographs in the "Drugs and the Pharmaceutical Sciences" series, Marcel Dekker.

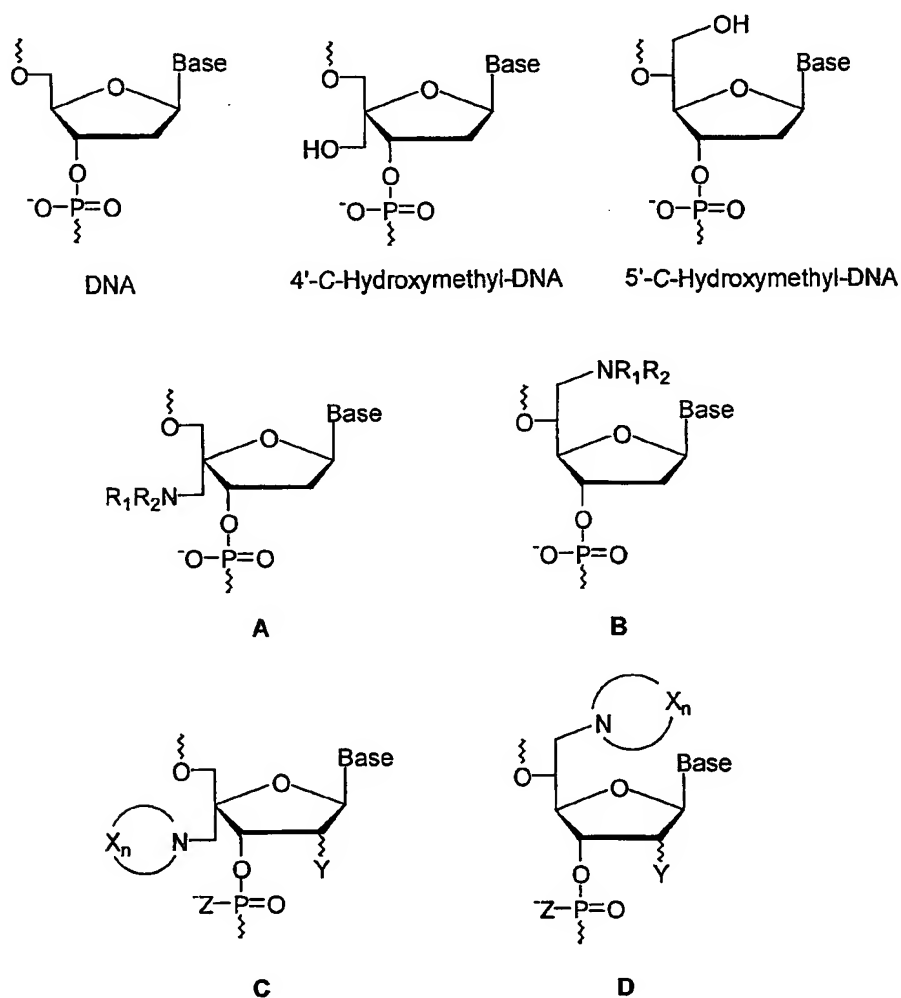
- In a further embodiment, the invention related to oligonucleotides of the invention which
15 further are covalently conjugated to a amino acid sequence, such as an enzyme or a active part thereof, particularly a oligonucleotides of the invention is covalently conjugated to a amino acid sequence or another moiety for enhanced cell membrane penetration and or Intra- or extracellular localization.

- 20 Finally the invention related to a method for producing a compound of formula I, which comprises reacting a compound of formula I, wherein the at least one of the substituents R^{4*} and R^{5*} contains a hydroxy group, with a optionally substituted non-aromatic ring containing at least one nitrogen atom a ring member, the substituents being defined above.

- 25 In addition, the nucleosides derivatives, nucleotide derivatives, oligonucleotides and polynucleotides of the invention can be prepared by methods well known to the skilled persons. Suitable methods are, inter alia, disclosed in the references mentioned herein, and in US 5,681,940, US 6,436,909, US 2002/0068708A1, US 2003/0092905, US
30 6,191,266, US 5,712,378, US 5,446,137, US 5,218,103, US 4,668,777, and in references mentioned in these articles, patents or applications.

- We disclose in a presently preferred embodiment, nucleosides and oligonucleotides containing 4'-C- and 5'-C-substituents composed of methyl groups functionalized with
35 cyclic saturated amines (see Figure 1, monomeric structures C and D), e.g., 4'-C- and 5'-C-piperidinomethyl, 4'-C- and 5'-C-piperazinomethyl or 4'-C- and 5'-C-(4-methylpiperazino)methyl groups. These derivatives are able to efficiently recognize complementary DNA or RNA target strands while obeying the Watson-Crick base-pairing rules. Key derivatives, e.g., 4'-C- and 5'-C-piperazinomethyl derivatives present a suitable
40 conjugation site, i.e., the N4 atom of the piperazino moiety, for attachment of a wide variety of substituents, reporter groups, reactive groups, transport-mediating groups etc. that can be attached either before or after oligomerization (oligonucleotide chain assembly). The novel derivatives are useful for diagnostic and therapeutic applications.

8

**Figure 1**

5 The oligonucleotides of the invention optionally contains, besides at least one nucleotide monomer of the invention, further nucleotide monomers selected from natural occurring nucleotides and analogues or mimics thereof. Examples are: DNA (A, T, G, C), RNA, LNA (Locked Nucleic Acids, such as beta-D-LNA, alpha-L-LNA, xylo-LNA, thio-LNA, 2'-amino-LNA), PNA (Peptide Nucleic Acid).

10

The monomers are coupled together via internucleoside linkages. Examples on such linkages are: phosphodiester, phosphorothioate, phosphotriester, phosphoramidate, methyl phosphonates, chain alkyl or cycloalkyl intersugar linkages or short chain heteroatomic or heterocyclic intersugar linkages. Presently a phosphorothioate linkage is

15

preferred. In other preferred embodiments, such as the peptide nucleic acid (PNA) backbone, the phosphodiester backbone of the oligonucleotide may be replaced with a polyamide backbone, the bases being bound directly or indirectly to the aza nitrogen

atoms of the polyamide backbone. P. E. Nielsen, M. Egholm, R. H. Berg, O. Buchardt, Science 1991, 254, 1497.

- Other preferred oligonucleotides may contain alkyl and halogen-substituted sugar moieties comprising one of the following at the 2' position: OH, SH, SCH₃, F, OCN, O(CH₂)_n-NH₂ or O(CH₂)_n-CH₃ where n is from 1 to about 10; C₁ to C₁₀ lower alkyl, substituted lower alkyl, alkaryl or aralkyl; Cl; Br; CN; CF₃; OCF₃; O-, S-, or N-alkyl; O-, S-, or N-alkenyl; SOCH₃; SO₂ CH₃; ONO₂; NO₂; N₃; NH₂; heterocycloalkyl; heterocycloalkaryl; aminoalkylamino; polyalkylamino; substituted silyl; an RNA cleaving group; a conjugate; a reporter group; an intercalator; a group for improving the pharmacokinetic properties of an oligonucleotide; or a group for improving the pharmacodynamic properties of an oligonucleotide and other substituents having similar properties. Oligonucleotides may also have sugar mimetics such as cyclobutyls in place of the pentofuranosyl group.

15 *Diagnostics*

- Several diagnostic and molecular biology procedures have been developed that utilise panels of different oligonucleotides to simultaneously analyse a target nucleic acid for the presence of a plethora of possible mutations. Typically, the oligonucleotide panels are immobilised in a predetermined pattern on a solid support such that the presence of a particular mutation in the target nucleic acid can be revealed by the position on the solid support where it hybridises. One important prerequisite for the successful use of panels of different oligonucleotides in the analysis of nucleic acids is that they are all specific for their particular target sequence under the single applied hybridisation condition. Since the affinity and specificity of standard oligonucleotides for their complementary target sequences depend heavily on their sequence and size this criteria has been difficult to fulfil so far.

- In a preferred embodiment, therefore, the nucleotides of the invention are used as a means to increase affinity and/or specificity of the probes and as a means to equalise the affinity of different oligonucleotides for their complementary sequences. As disclosed herein such affinity modulation can be accomplished by, e.g., replacing selected nucleosides in the oligonucleotide with a nucleotide of the invention carrying a similar nucleobase.

- In another preferred embodiment the high affinity and specificity of modified oligonucleotides is exploited in the sequence specific capture and purification of natural or synthetic nucleic acids. In one aspect, the natural or synthetic nucleic acids are contacted with the modified oligonucleotide immobilised on a solid surface. In this case hybridisation and capture occurs simultaneously. The captured nucleic acids may be, for instance, detected, characterised, quantified or amplified directly on the surface by a variety of methods well known in the art or it may be released from the surface, before such characterisation or amplification occurs, by subjecting the immobilised, modified oligonucleotide and captured nucleic acid to dehybridising conditions, such as for example heat or by using buffers of low ionic strength.

The solid support may be chosen from a wide range of polymer materials such as for instance CPG (controlled pore glass), polypropylene, polystyrene, polycarbonate or polyethylene and it may take a variety of forms such as for instance a tube, a micro-titer plate, a stick, a bead, a filter, etc.. The modified oligonucleotide may be immobilised to the solid support via its 5' or 3' end (or via the terminus of linkers attached to the 5' or 3' end) by a variety of chemical or photochemical methods usually employed in the immobilisation of oligonucleotides or by non-covalent coupling such as for instance via binding of a biotinylated modified oligonucleotide to immobilised streptavidin. One preferred method for immobilising modified oligonucleotides on different solid supports is photochemical using a photochemically active anthraquinone covalently attached to the 5'- or 3'-end of the modified oligonucleotide (optionally via linkers) as described in (WO 96/31557). Thus, the present invention also provide a surface carrying an modified oligonucleotide.

In another aspect the modified oligonucleotide carries a ligand covalently attached to either the 5'- or 3'-end. In this case the modified oligonucleotide is contacted with the natural or synthetic nucleic acids in solution whereafter the hybrids formed are captured onto a solid support carrying molecules that can specifically bind the ligand.

In still another aspect, modified oligonucleotides capable of performing "strand displacement" are used in the capture of natural and synthetic nucleic acids without prior denaturation. Such modified oligonucleotides are particularly useful in cases where the target sequence is difficult or impossible to access by normal oligonucleotides due to the rapid formation of stable intramolecular structures. Examples of nucleic acids comprising such structures are double stranded DNA, rRNA, tRNA, snRNA and scRNA.

In another preferred embodiment, modified oligonucleotides designed with the purpose of high specificity are used as primers in the sequencing of nucleic acids and as primers in any of the several well known amplification reactions, such as the PCR reaction. As shown herein, the design of the modified oligonucleotides determines whether it will sustain an exponential or linear target amplification. The products of the amplification reaction can be analysed by a variety of methods applicable to the analysis of amplification products generated with normal DNA primers. In the particular case where the modified oligonucleotide primers are designed to sustain a linear amplification the resulting amplicons will carry single stranded ends that can be targeted by complementary probes without denaturation. Such ends could for instance be used to capture amplicons by other complementary modified oligonucleotides attached to a solid surface.

In another aspect, modified oligonucleotides capable of "strand displacement" are used as primers in either linear or exponential amplification reactions. The use of such oligonucleotides is expected to enhance overall amplicon yields by effectively competing with amplicon re-hybridisation in the later stages of the amplification reaction. Demers, et al. (*Nucl. Acid Res.* 1995, Vol 23, 3050-3055) discloses the use of high-affinity, non-extendible oligomers as a means of increasing the overall yield of a PCR reaction. It is believed that the oligomers elicit these effects by interfering with amplicon re-hybridisation in the later stages of the PCR reaction. It is expected that modified oligonucleotides

blocked at their 3' end will provide the same advantage. Blocking of the 3' end can be achieved in numerous ways like for instance by exchanging the 3' hydroxyl group with hydrogen or phosphate. Such 3' blocked modified oligonucleotides can also be used to selectively amplify closely related nucleic acid sequences in a way similar to that described
5 by Yu et al. (*Biotechniques*, 1997, **23**, 714-716).

In recent years, novel classes of probes that can be used in for example real-time detection of amplicons generated by target amplification reactions have been invented. One such class of probes have been termed "Molecular Beacons". These probes are
10 synthesised as partly self-complementary oligonucleotides comprising a fluorophor at one end and a quencher molecule at the other end. When free in solution the probe folds up into a hairpin structure (guided by the self-complimentary regions) which positions the quencher in sufficient closeness to the fluorophor to quench its fluorescent signal. Upon hybridisation to its target nucleic acid, the hairpin opens thereby separating the fluorophor
15 and quencher and giving off a fluorescent signal.

Another class of probes have been termed "Taqman probes". These probes also comprise a fluorophor and a quencher molecule. Contrary to the Molecular Beacons, however, the quenchers ability to quench the fluorescent signal from the fluorophor is maintained after
20 hybridisation of the probe to its target sequence. Instead, the fluorescent signal is generated after hybridisation by physical detachment of either the quencher or fluorophor from the probe by the action of the 5' exonuclease activity of a polymerase which has initiated synthesis from a primer located 5' to the binding site of the Taqman probe.

25 High affinity for the target site is an important feature in both types of probes and consequently such probes tends to be fairly large (typically 30 to 40 mers). As a result, significant problems are encountered in the production of high quality probes. In a preferred embodiment, therefore, the nucleotide of the invention is used to improve production and subsequent performance of Taqman probes and Molecular Beacons by
30 reducing their size whilst retaining the required affinity.

In a further aspect, the nucleotides of the invention are used to construct new affinity pairs (either fully or partially modified oligonucleotides). The affinity constants can easily be adjusted over a wide range and a vast number of affinity pairs can be designed and
35 synthesised. One part of the affinity pair can be attached to the molecule of interest (e.g. proteins, amplicons, enzymes, polysaccharides, antibodies, haptens, peptides, PNA, etc.) by standard methods, while the other part of the affinity pair can be attached to e.g. a solid support such as beads, membranes, micro-titer plates, sticks, tubes, etc. The solid support may be chosen from a wide range of polymer materials such as for instance
40 polypropylene, polystyrene, polycarbonate or polyethylene. The affinity pairs may be used in selective isolation, purification, capture and detection of a diversity of the target molecules mentioned above.

The principle of capturing a nucleotide-tagged molecule by ways of interaction with another complementary oligonucleotide (either fully or partially modified) can be used to create an infinite number of novel affinity pairs.

- 5 In another preferred embodiment the high affinity and specificity of modified oligonucleotides are exploited in the construction of probes useful in *in-situ* hybridisation. For instance, could be used to reduce the size of traditional DNA probes while maintaining the required affinity thereby increasing the kinetics of the probe and its ability to penetrate the sample specimen.

10

In another preferred embodiment, modified oligonucleotides to be used in antisense therapeutics are designed with the dual purpose of high affinity and ability to recruit RNaseH. This can be achieved by, for instance, having segments flanking an unmodified central DNA segment.

15

Definitions

DMTr=4,4'-dimethoxytrityl

CEPA=2-cyanoethyl-(N,N'-diisopropyl)phosphoramido

TBDMS=t-Butyldimethylsilyl

20 Ac=acetyl

TBDMSM=t-butyldimethylsiloxyethyl

N₃=azido

OTs=tosyl

ON = oligonucleotide

25

The term "at least one" includes integers greater or equal to 1, such as 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 50, 100, or more.

In the formulas, the substituents have the same meanings as in the IUPAC Compendium of
30 Chemical Terminology unless otherwise defined. When the substituent definition comprises a range (e.g. ; C₁ to C₁₂ or C₁ to C₆), the range is understood to comprise all integers in that range, i.e. 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22 etc.

- 35 The term "substituted" means that one or more (such as 1, 2, 3, 4, 5, or 6) hydrogen atoms are substituted with substituents independently selected from groups such as: halogen atoms, nitro groups, hydroxyl, mercapto, cyano, carbamoyl, optionally substituted amino, optionally substituted alkyl (e.g. perhalogenalkyl), optionally substituted alkenyl, optionally substituted alkynyl, optionally substituted cycloalk(en/yn)yl, optionally
40 substituted aryl, optionally substituted alkoxy, optionally substituted aryloxy, optionally substituted alkoxy, optionally substituted alkylthio, optionally substituted (hetero)aryl, optionally substituted (hetero)aryloxy or acyl groups.

The term "halogen" represents fluoro, chloro, bromo, or iodo.

45

The term "heteroatom" includes atoms such as O, S, or N.

The term "alkyl" includes straight or branched chain aliphatic hydrocarbon groups that are saturated and have 1 to 15 carbon atoms. Preferably, the alkyl group have 1-10 carbon atoms, and most preferred 1, 2, 3, 4, 5, or 6 carbon atoms. The alkyl groups may be interrupted by one or more heteroatoms, and may be substituted, e.g. with groups as defined above, such as halogen, hydroxyl, aryl, cycloalkyl, aryloxy, or alkoxy. Preferred straight or branched alkyl groups include methyl, ethyl, propyl, isopropyl, butyl and t-butyl. Alkyl includes cycloalkyl.

10

The term "cycloalkyl" includes straight or branched chain, saturated or unsaturated aliphatic hydrocarbon groups which connect to form one or more rings of preferably 3, 4, 5, 6, or 7 ring members, which can be fused or isolated. The rings may be substituted, e.g. with groups as defined above, such as halogen, hydroxyl, aryl, aryloxy, alkoxy, or alkyl.

15 Preferred cycloalkyl groups include cyclopropyl, cyclobutyl, cyclopentyl and cyclohexyl.

The term "alkenyl" includes straight or branched chain hydrocarbon groups having 2 to 15 carbon atoms (e.g. 2, 3, 4, 5, 6 or 10 carbon atoms) with at least one carbon-carbon double bond, the chain being optionally interrupted by one or more heteroatoms. The chain hydrogens may be substituted, e.g. with groups as defined above, such as halogen. Preferred straight or branched alkenyl groups include vinyl, allyl, 1-butenyl, 1-methyl-2-propenyl and 4-pentenyl.

The term "alkynyl" includes straight or branched chain hydrocarbon groups having 2 to 15 carbon atoms (e.g. 2, 3, 4, 5, 6 or 10 carbon atoms) with at least one carbon-carbon triple bond, the chain being optionally interrupted by one or more heteroatoms. The chain hydrogens may be substituted, e.g. with groups as defined above, such as halogen. Preferred straight or branched alkynyl groups include ethynyl, propynyl, 1-butylnyl, 1-methyl-2-propynyl and 4-pentylnyl.

30

The term "aryl" refers to carbon-based rings which are aromatic. The rings may be isolated, such as phenyl, or fused, such as naphthyl. The ring hydrogens may be substituted, e.g. with groups as defined above, such as alkyl, halogen, free or functionalized hydroxy, trihalomethyl, etc. Preferred aryl groups include phenyl, 3-(trifluoromethyl)phenyl, 3-chlorophenyl, and 4-fluorophenyl.

35

The term "heterocyclyl" is intended to mean a aromatic (heteroaryl) or non-aromatic carbocyclic ring or ring system where one or more of the carbon atoms have been replaced with heteroatoms, e.g. nitrogen (=N- or -NH-), sulphur, and/or oxygen atoms. Examples of such heterocyclyl groups are imidazolidine, piperazine, hexahydropyridazine, hexahydropyrimidine, diazepane, diazocane, pyrrolidine, piperidine, azepane, azocane, aziridine, azirine, azetidine, pyrroline, tropane, oxazoline (morpholine), azepine, dihydroazepine, tetrahydroazepine, and hexahydroazepine, oxazolane, oxazepane, oxazocane, thiazolane, thiazinane, thiazepane, thiazocane, oxazetane, diazetane, thiazetane, tetrahydrofuran, tetrahydropyran, oxepane, tetrahydrothiophene,

45

tetrahydrothiopyrane, thiepane, dithiane, dithiepane, dioxane, dioxepane, oxathiane, oxathiepane. The most interesting examples are imidazolidine, piperazine, hexahydropyridazine, hexahydropyrimidine, diazepane, diazocane, pyrrolidine, piperidine, azepane, azocane, azetidine, tropane, oxazinane (morpholine), oxazolane, oxazepane, 5 thiazolane, thiazinane, and thiazepane, in particular imidazolidine, piperazine, hexahydropyridazine, hexahydropyrimidine, diazepane, pyrrolidine, piperidine, azepane, oxazinane (morpholine), and thiazinane. Examples of aromatic rings are pyridine, pyridazine, pyrimidine, pyrazine, triazine, thiophene, oxazole, isoxazole, thiazole, isothiazole, pyrrole, imidazole, pyrazole, tetrazole, quinoline, benzothiazole, benzotriazole, 10 benzodiazole, benzoxazole, triazole, isoquinoline, indole, benzopyrazole, thiadiazole, and oxadiazole. The presently most interesting examples of aromatic rings are pyridine, pyridazine, pyrimidine, pyrazine, thiophene, tetrazole, oxazole, isoxazole, thiazole, isothiazole, pyrrole, imidazole, pyrazole, quinoline, triazole, isoquinoline, and indole, in particular pyridine, thiophene, imidazole, quinoline, isoquinoline, indole, and tetrazole.

15

The term nitrogen containing non-aromatic heterocyclic ring is meant to comprise any ring system as above defined, which comprise at least one nitrogen atom in the ring system, and the ring system can be substituted with at least one substituent as defined above. Presently preferred is saturated ring systems comprising one or two nitrogen atoms and 4 20 or 5 carbon atoms in the same ring. A presently preferred ring is depicted in fig 1, C and D, wherein X_n stands for an alkylene chain, optionally interrupted by a nitrogen atom (which can be substituted with alkyl).

The term "heteroaryl" refers to aromatic rings (having such as 3, 4, 5, 6, or 7 ring 25 members) which contain at least one (e.g. 1, 2, 3, 4, or 5) heteroatom(s) in the ring. Heteroaryl rings may be isolated, preferably with 5 to 6 ring atoms, or fused, preferably with 8, 9 or 10 ring atoms. The heteroaryl ring(s) hydrogens or heteroatoms with open valency may be substituted, e.g. with groups as defined above, such as alkyl or halogen. Examples of heteroaryl groups include imidazole, pyridine, indole, quinoline, furan, 30 thiophene, pyrrole, tetrahydroquinoline, dihydrobenzofuran, and dihydrobenzindole.

The term "acyl" encompasses carboxylic acyl groups having the formula $A-C(=O)-$, in which formula A represents a substituent as defined above, such as an alkyl, alkenyl, aryl, heterocyclyl or aralkyl group, the chain in said groups being optionally interrupted by one 35 or more heteroatoms and the groups being optionally substituted, e.g. by one or more substituents as defined above. Examples on acyl groups are formyl, C_{1-6} -alk(en/yn)ylcarbonyl, arylcarbonyl, aryl- C_{1-6} alk(en/yn)ylcarbonyl, cycloalkylcarbonyl, or cycloalkyl- C_{1-6} alk(en/yn)ylcarbonyl group. Also, the term acyl comprises any of the above groups in which the $C(=O)$ group is replaced by $C(=S)$ or $C(=N-R)$, R is H or a substituent 40 as defined above.

The terms "pharmaceutically acceptable salt" and "pharmaceutical carrier" are well known to the person skilled in the art.

The term "salts" is intended to include pharmaceutically acceptable acid addition salts obtainable by treating the base form of a functional group, such as an amine, with appropriate acids such as inorganic acids, for example hydrohalic acids; typically hydrochloric, hydrobromic, hydrofluoric, or hydroiodic acid; sulfuric acid; nitric acid; phosphoric acid and the like; or organic acids, for example acetic, propionic, hydroacetic, 2-hydroxypropanoic acid, 2-oxopropanoic acid, ethandioic, propanedioic, butanedioic, (Z)-2-butenedioic, (E)-butenedioic, 2-hydroxybutanedioic, 2,3-dihydroxybutanedioic, 2-hydroxy-1,2,3-propanetricarboxylic, methanesulfonic, ethanesulfonic, benzenesulfonic, 4-methylbenzenesulfonic acid, cyclohexanesulfamic, 2-hydroxybenzoic, 4-amino-2-hydroxybenzoic, and other acids known to the skilled practitioner. Further examples are the iodide, acetate, phenylacetate, trifluoroacetate, acrylate, ascorbate, benzoate, chlorobenzoate, dinitrobenzoate, hydroxybenzoate, methoxybenzoate, methylbenzoate, o-acetoxybenzoate, naphthalene-2-benzoate, bromide, isobutyrate, phenylbutyrate, g-hydroxybutyrate, b-hydroxybutyrate, butyne-1,4-dioate, hexyne-1,4-dioate, hexyne-1,6-dioate, caproate, caprylate, chloride, cinnamate, citrate, decanoate, formate, fumarate, glycollate, heptanoate, hippurate, lactate, malate, maleate, hydroxymaleate, malonate, mandelate, mesylate, nicotinate, isonicotinate, nitrate, oxalate, phthalate, terephthalate, phosphate, monohydrogenphosphate, dihydrogenphosphate, metaphosphate, pyrophosphate, propiolate, propionate, phenylpropionate, salicylate, sebacate, succinate, suberate, sulfate, bisulfate, pyrosulfate, sulfite, bisulfite, sulfonate, benzenesulfonate, p-bromophenylsulfonate, chlorobenzenesulfonate, propanesulfonate, ethanesulfonate, 2-hydroxyethanesulfonate, methanesulfonate, naphthalene-1-sulfonate, naphthalene-2-sulfonate, p-toluenesulfonate, xylenesulfonate, tartarate, and the like of a oligonucleotide or nucleotide derivative of the invention.

25

The term "base addition salts" include alkali metals, such as sodium and potassium, alkali earth metals, such as calcium and magnesium, and organic addition salts such as quaternary ammonium cations.

It should furthermore be understood that the compounds defined herein include possible salts thereof, of which pharmaceutically acceptable salts are of course especially relevant for the therapeutic applications. Salts include acid addition salts and basic salts. Examples of acid addition salts are hydrochloride salts, fumarate, oxalate, etc. Examples of basic salts are salts where the (remaining) counter ion is selected from alkali metals, such as sodium and potassium, alkaline earth metals, such as calcium salts, potassium salts, and ammonium ions ($^+N(R')_4$, where the R's independently designates optionally substituted C_{1-6} -alkyl, optionally substituted C_{2-6} -alkenyl, optionally substituted aryl, or optionally substituted heteroaryl). Pharmaceutically acceptable salts are, e.g., those described in Remington's - The Science and Practice of Pharmacy, 20th Ed. Alfonso R. Gennaro (Ed.), Lippincott, Williams & Wilkins; ISBN: 0683306472, 2000, and in Encyclopedia of Pharmaceutical Technology. However, generally preferred salt forming agents for application in the present invention are organic dicarboxylic acids such as oxalic, fumaric, and maleic acid, and the like.

45 The term "nucleoside," as used herein, refers to a compound comprising a purine or

pyrimidine base (or derivative thereof) covalently joined to a 5 atom cyclic sugar (furanose), e.g. ribose, 2'-deoxyribose, and 2',3'-dideoxyribose, or analogues thereof where O in the ribose is replaced with another group, such as S or NH. The term "nucleoside" is used broadly so as to include the sugar modified nucleosides of the invention.

The term "polynucleotide," as used herein, refers to polymers comprising of two or more nucleoside moieties, wherein each nucleoside moiety is joined to one (terminal) or two (internal) other nucleoside moieties through Internucleoside linkages such as phosphodiester linkages, peptide linkages, phosphonate linkages, phosphoramidate, phosphorothioate linkages, and the like. RNA and DNA are examples of polynucleotides. The term "polynucleotide", as used herein, unless noted otherwise, is used broadly so as to include the sugar modified polynucleotides of the invention.

The term "oligonucleotide", as used herein, is to refer to relatively small polynucleotides, e.g. polynucleotides of between 2 and about 50 base pairs in length; however the oligonucleotide of the invention may be significantly longer.

The terms "hydroxyl blocking group" and "OH protection group" as used herein is readily understood by the person of ordinary skill in the art of organic chemistry, examples of hydroxyl blocking groups, and other blocking groups, can be found (among other places) in Greene and Wuts, "Protective Groups In Organic Synthesis" John Wiley & Sons, NY, N.Y. (1991).

The terms "base" and nucleoside base" as used herein refer to heterocyclic nucleotide bases found in naturally occurring nucleic acid such as adenine, cytosine, hypoxanthine, uracil, thymine, guanine and analogs thereof, including non-naturally occurring bases that are capable of forming base-pairing relationships with naturally occurring nucleotide bases. Such non-naturally occurring heterocyclic bases include, but are not limited to, aza and deaza pyrimidine analogs, aza and deaza purine analogs as well as other heterocyclic base analogs, wherein one or more of the carbon and nitrogen atoms of the purine and pyrimidine rings have been substituted by heteroatoms, e.g. oxygen, sulfur, selenium, phosphorus, and the like. Thus, "nucleobase" includes not only the known purine and pyrimidine heterocycles, but also heterocyclic analogues and tautomers thereof. Illustrative examples of nucleobases are adenine, guanine, thymine, cytosine, uracil, purine, xanthine, diaminopurine, 8-oxo-N⁶-methyladenine, 7-deazaxanthine, 7-deazaguanine, N⁴,N⁴-ethanocytosin, N⁶,N⁶-ethano-2,6-diaminopurine, 5-methylcytosine, 5-(C³-C⁶)-alkynyl-cytosine, 5-fluorouracil, 5-bromouracil, pseudoisocytosine, 2-hydroxy-5-methyl-4-triazolopyridin, isocytosine, isoguanin, inosine, N⁶-allyl purines, N⁶-acyl purines, N⁶-benzylpurine, N⁶-halopurine, N⁶-vinylpurine, N⁶-acetylenic purine, N⁶-acyl purine, N⁶-hydroxyalkyl purine, N⁶-thioalkyl purine, N²-alkyl purines, N⁴-alkylpyrimidines, N⁴-acylpyrimidines, N⁴-benzylpurine, N⁴-halopyrimidines, N⁴-vinylpyrimidines, N⁴-acetylenic pyrimidines, N⁴-acyl pyrimidines, N⁴-hydroxyalkyl pyrimidines, N⁶-thioalkyl pyrimidines, 6-azapyrimidine, including 6-azacytosine, 2- and/or 4- mercaptopyrimidine, uracil, C⁵-alkylpyrimidines, C⁵-benzylpyrimidines, C⁵-halopyrimidines, C⁵-vinylpyrimidine, C⁵-

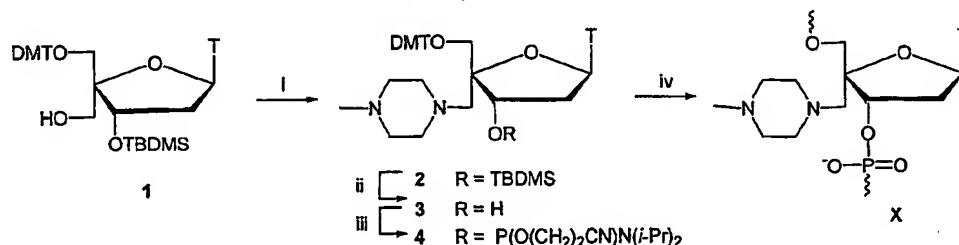
acetylenic pyrimidine, C⁵-acyl pyrimidine, C⁵-hydroxyalkyl purine, C⁵-amidopyrimidine, C⁵-cyanopyrimidine, C⁵-nitropyrimidine, C⁵-aminopyrimidine, N²-alkylpurines, N²-alkyl-6-thiopurines, 5-azacytidinyl, 5-azauracilyl, trazolopyridinyl, imidazolopyridinyl, pyrrolopyrimidinyl, and pyrazolopyrimidinyl. Functional oxygen and nitrogen groups on the base can be protected and deprotected if necessary or desirable. Suitable protecting groups are well known to those skilled in the art, and included trimethylsilyl, dimethylhexylsilyl, *t*-butyldimethylsilyl, and *t*-butyldiphenylsilyl, trityl, alkyl groups, acyl groups such as acetyl and propionyl, methanesulfonyl, and *p*-toluenesulfonyl. Preferred bases include adenine, guanine, 2,6-diaminopurine, thymine, 2-thiothymine, cytosine, methyl cytosine, uracil, 5-fluorocytosine, xanthine, 6-aminopurine, 2-aminopurine, 6-chloro-2-amino-purine, and 6-chloropurine. Especially interesting nucleobases are adenine, guanine, thymine, cytosine, and uracil, which are considered as the naturally occurring nucleobases in relation to therapeutic and diagnostic application in humans.

15

Synthesis of phosphoramidite building blocks

Synthesis of phosphoramidite derivative **4** is depicted in Scheme 1. The 4'-C-hydroxymethyl group of the known nucleoside **1**¹⁷ was converted into the 4'-C-(4-methylpiperazino)methyl group by reacting with trifluoromethanesulfonic anhydride in anhydrous pyridine followed by treatment with 10 equivalents of N-methylpiperazine in anhydrous THF to give nucleoside **2**. The *tert*-butyldimethylsilyl group was removed to give nucleoside **3** which was phosphitylated at the 3'-hydroxy group to furnish the phosphoramidite **4** in an overall yield of 27% (from **1**). Introduction of other cyclic saturated amino groups can be achieved by substituting 4-methylpiperazine (in the transformation of nucleoside **1** to nucleoside **2**) with other cyclic amines like optionally substituted aziridine, optionally substituted azetidine, optionally substituted pyrrolidine, optionally substituted piperidine, optionally substituted piperazine, optionally substituted and appropriately protected piperazine, optionally substituted thiazolidine, optionally substituted thiazine, optionally substituted pyrazolidine, optionally substituted morpholine, optionally substituted thiomorpholine, optionally substituted crown ether moieties, and other optionally substituted monocyclic or polycyclic saturated amines; in all cases with appropriate protecting groups optionally installed in order to protect reactive groups and/or atoms in the cyclic saturated amine (e.g., other nitrogen atom(s) than the nitrogen atom functioning as the nucleophilic atom in the transformation of nucleoside **1** to nucleoside **2**). The example synthesis depicted in Scheme 1 contains thymine as nucleobase. Similar transformations as those described above using starting nucleosides corresponding to nucleoside **1** but containing other optionally substituted and optionally protected nucleobases, e.g., uracil, 4-*N*-benzoylcytosine, 4-*N*-acetylcytosine, 6-*N*-benzoyladenine or 2-*N*-isobutyrylguanine, can be used for preparation of phosphoramidite derivatives corresponding to amidite **4** but with other optionally substituted and optionally protected nucleobases, e.g., uracil, 4-*N*-benzoylcytosine, 4-*N*-acetylcytosine, 6-*N*-benzoyladenine or 2-*N*-isobutyrylguanine. Similarly, using starting nucleosides known from the literature¹¹⁻¹⁵ and procedures as those described above, a person skilled in the art will be able to synthesize the corresponding 5'-C-substituted derivatives (see Figure 1,

monomeric structure **D**) with 5'-C-substituents composed of methyl groups functionalized with cyclic saturated amines.

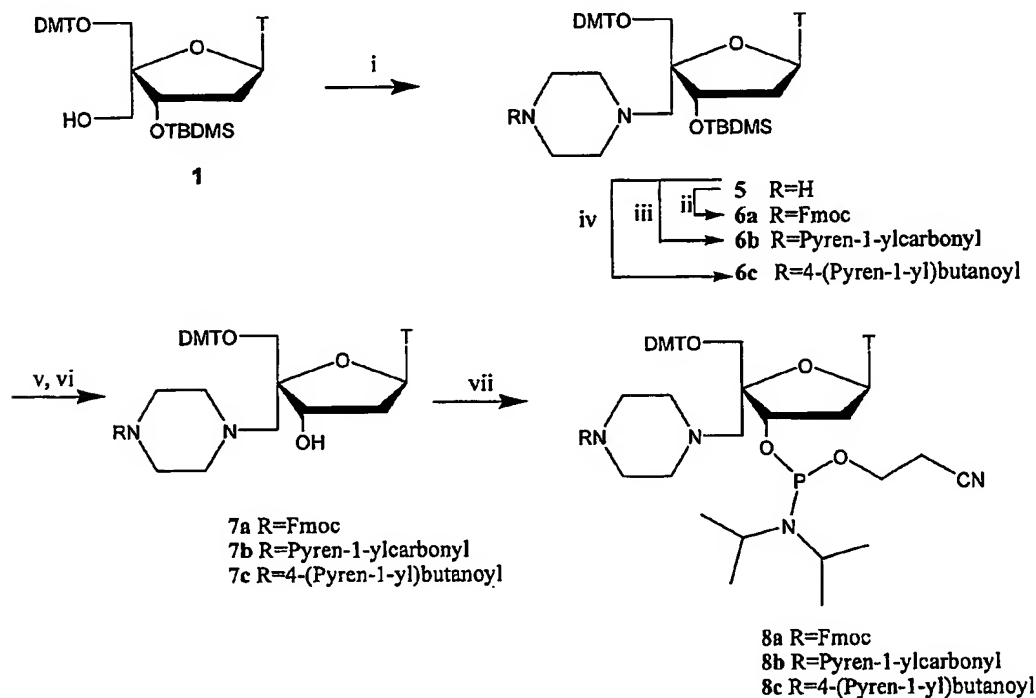


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Scheme 1. Reagents and conditions: i) a) TF_2O , anhydrous pyridine, CH_2Cl_2 , b) 4-methylpiperazine, anhydrous THF (54%); ii) TBAF, THF (77%); iii) 2-cyanoethyl *N,N*-diisopropylphosphoramido-chloridite, DIPEA, CH_2Cl_2 , r.t., 63%; iv) DNA synthesizer. T = thymine-1-yl, DMT = 4,4'-dimethoxytrityl.

Synthesis of phosphoramidites **8a**, **8b** and **8c** are depicted in Scheme 2. Nucleoside **5** was obtained by reaction of nucleoside **1** by reaction with trifluoromethanesulfonic anhydride in anhydrous pyridine followed by treatment with piperazine in anhydrous THF. Subsequent reactions with 9-fluorenylmethyl chloroformate in anhydrous pyridine, 1-pyrenecarboxylic acid and *N,N*-diethylcarbodiimide, or 1-pyrenebutanoic acid and *N,N*-diethylcarbodiimide furnished the *N*-derivatized compounds **6a**, **6b** and **6c**, respectively. Subsequent desilylation to give nucleosides **7a**, **7b** and **7c**, respectively, followed by phosphorylation afforded the phosphoramidite building blocks **8a**, **8b** and **8c**, respectively. Introduction of other cyclic saturated amino groups can be achieved by substituting 4-(methyl)piperazine (in the transformation of nucleoside **1** to nucleoside **2**) with other cyclic amines like optionally substituted aziridine, optionally substituted azetidine, optionally substituted pyrrolidine, optionally substituted piperidine, optionally substituted piperazine, optionally substituted and appropriately protected piperazine, optionally substituted thiazolidine, optionally substituted thiazine, optionally substituted pyrazolidine, optionally substituted morpholine, optionally substituted thiomorpholine, optionally substituted crown ether moieties, and other optionally substituted monocyclic or polycyclic saturated amines; in all cases with appropriate protecting groups optionally installed in order to protect reactive groups and/or atoms in the cyclic saturated amine (e.g., other nitrogen atom(s) than the nitrogen atom functioning as the nucleophilic atom in the transformation of nucleoside **1** to nucleoside **2**). The example synthesis depicted in Scheme 1 contains thymine as nucleobase. Similar transformations as those described above using starting nucleosides corresponding to nucleoside **1** but containing other optionally substituted and optionally protected nucleobases, e.g., uracil, 4-*N*-benzoylcytosine, 4-*N*-acetylcytosine, 6-*N*-benzoyladenine or 2-*N*-isobutyrylguanine, can be used for preparation of phosphoramidite

derivatives corresponding to amidite 4 but with other optionally substituted and optionally protected nucleobases, *e.g.*, uracil, 4-*N*-benzoylcytosine, 4-*N*-acetylcytosine, 6-*N*-benzoyladenine or 2-*N*-isobutyrylguanine. Similarly, using starting nucleosides known from the literature¹¹⁻¹⁵ and procedures as those described above, a person skilled in the art will be able to synthesize the corresponding 5'-C substituted derivatives (see Figure 1, monomeric structure **D**) with substituents composed of methyl groups functionalized with cyclic saturated amines.



10

Scheme 2. i) a) TiF_2O , anhydrous pyridine, CH_2Cl_2 , b) piperazine, anhydrous THF; ii) 9-fluorenylmethyl chloroformate, anhydrous pyridine; iii) 1-pyrenecarboxylic acid, *N,N*-diethylcarbodiimide, anhydrous CH_2Cl_2 ; iii) 4-(pyren-1-yl)butanoic acid, *N,N*-diethylcarbodiimide, anhydrous CH_2Cl_2 ; v) triethylamine tris-hydrofluoride, pyridine hydrochloride, THF; vi) TBAF, THF; vii) 2-cyanoethyl *N,N*-diisopropylphosphoramidochloridite, *N,N*-diisopropylethylamine.

Synthesis of oligonucleotides

The DNA references **ON1** and **ON4**, the LNA reference **ON5** and the functionalized (containing monomer **X** derived from incorporation of amidite **4**) ONs **ON2**, **ON3**, **ON6**, **ON7** and **ON8** (Tables 1 and 2) were synthesized in 0.2 μmol scale on an automated DNA synthesizer using standard DNA and LNA amidites, amidite **4** (>90% coupling yield, 10 min coupling time using 1*H*-tetrazole as activator and standard iodine oxidation). Satisfactory purities (>80 %) of all functionalized ONs were verified by capillary gel electrophoresis and the compositions by MALDI-MS analysis (MALDI-MS; selected data:

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m/z ($[M-H]^+$ found/calcd.) 2863/2866 (**ON2**) and 3089/3090 (**ON3**). Similar coupling conditions using appropriately protected 4'-C- or 5'-C-substituted amidites can be used to prepare the other 4'-C- or 5'-C-substituted ONs of this invention, *i.e.*, ONs containing monomers **C** or **D** (see Figure 1).

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The phosphoramidite building blocks **8a**, **8b** and **8c** were used to synthesize **ON9-ON14** and **ON16-ON21** (Table 4) and **ON22-ON25** (Table 5) on an automated DNA synthesizer. The coupling yields of **8a**, **8b** and **8c** were 98%, 90% and 98% (10 min coupling time, 1*H*-tetrazole as activator), respectively, and ~99% for unmodified DNA phosphoramidites (2 min coupling time, 1*H*-tetrazole as activator). Amidite **8a** was applied for synthesis of various functionalized ONs (see experimentals), including several of the ONs shown in Tables 4 and 4, employing an on-column conjugation approach involving selective removal of the Fmoc groups and subsequent reaction with, *e.g.*, 1-pyrenecarboxylic acid and HBTU in DMF following essentially a procedure published for synthesis of 5'-end conjugated ONs.²¹ The composition of the synthesized ONs was verified by MALDI-MS[†] and the purity (>80%) by capillary gel electrophoresis. MALDI-MZ m/z ($[M-H]^+$ found/calcd.) 2852/2853 (**ON9**), 3122/3125 (**ON10**), 2879/2878 (**ON11**), 3336/3332 (**ON12**), 3048/3048 (**ON13**), 3730/3731 (**ON14**), 3061/3059 (**ON16**), 3387/3388 (**ON17**), 3387/3387 (**ON18**).

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Hybridization studies

The hybridization properties of the functionalized ONs **ON2**, **ON3**, **ON6**, **ON7** and **ON8** (Tables 1 and 2) were evaluated by thermal denaturation studies, and the changes in melting temperature (ΔT_m values) relative to the T_m values obtained for the relevant reference ON were calculated (Tables 1 and 2). Towards the DNA complement in the medium salt buffer, **ON2** and **ON3** containing 4'-C-(*N*-methylpiperazino)methyl-DNA monomer **X** displayed significantly increased T_m values relative to reference **ON1**. The corresponding T_m values towards the RNA complement were unchanged compared with the reference values. An additional affinity-enhancing effect was seen when changing to the low salt buffer (Table 1). In comparison with the corresponding 4'-C-hydroxymethyl-DNA monomer,¹⁷ similar 4'-C-aminoalkyl-DNA monomers,⁸⁻¹⁰ and a biotinylated 4'-C-alkyl monomer,¹⁹ 4'-C-*N*-(methylpiperazino)methyl monomer **X** displays the most significant affinity-enhancing effect towards a DNA complement. Importantly, the Watson-Crick base pairing rules are obeyed as shown in Table 3. It is noteworthy that **ON3**, especially at low salt conditions, displays a clear thermal preference of hybridizing towards DNA rather than RNA. These data indicate that the basic piperazino group of monomer **X** is at least partly protonated at pH 7.0 leading to a favorable effect of partial charge neutralization at reduced salt concentrations at which anion shielding by sodium ions is weakened. Similar effects as described above are seen for **ON6** and **ON7** relative to the corresponding DNA reference **ON4** and LNA reference **ON5**, respectively (Table 2). Thus, both when incorporated into a DNA strand or into an RNA mimicking LNA strand, monomer **X** induces increased thermal stability of duplexes towards DNA and has no significant effect on the

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thermal stability of duplexes towards RNA. This general trend is conformed for **ON8**. The obtained data reveal that the novel ONs of this invention containing monomers **C** or **D** (see Figure 1) induced increased binding affinity towards complementary single-stranded target strands, especially towards DNA. Furthermore it is shown that this effect is more pronounced when lowering the salt concentration of the hybridization buffer.

10 **Table 1.** T_m values towards DNA/RNA complements.^a

Sequence	110 mM Na ⁺		40 mM Na ⁺	
	DNA target	RNA target	DNA target	RNA target
	$T_m (\Delta T_m)/^{\circ}\text{C}$	$T_m (\Delta T_m)/^{\circ}\text{C}$	$T_m (\Delta T_m)/^{\circ}\text{C}$	$T_m (\Delta T_m)/^{\circ}\text{C}$
5'-d(GTGATATGC) (ON1)	29	26 ^b /28 ^c	22	19 ^b /20 ^c
5'-d(GTGAXATGC) (ON2)	33 (+4)	28 ^c (0)	26 (+4)	21 ^c (+1)
5'-d(GXGAXXGC) (ON3)	35 (+6)	29 ^c (+1)	31 (+9)	22 ^c (+2)

^aMelting temperatures [T_m values (ΔT_m values are calculated relative to the T_m value of the reference ON (here **ON1**)] measured as the maximum of the first derivative of the melting curve (A_{260} vs. temperature; 10 °C to 80 °C with an increase of 1 °C/min) recorded in medium salt buffer ["110 mM Na⁺"] (10 mM sodium phosphate, 100 mM sodium chloride, pH 7.0) and low salt buffer ["40 mM Na⁺"] (10 mM sodium phosphate, 30 mM sodium chloride, pH 7.0) using 1 μM concentrations of the two complementary strands.

^{b,c}Determined in two different experimental series.

20 Incorporation of one, two or three 4'-C-piperazinomethyl monomer(s) Y (Table 4) induce a small increase in the thermal denaturation temperature (T_m value) with ΔT_m values (change in T_m value *per modification*) of approximately +2 °C towards the DNA complement and virtually no change in the T_m value towards the RNA target. These data are similar to those obtained for the corresponding 4'-C-(N-methyl)piperazinomethyl monomer X. The tendency towards DNA-selective hybridization observed for ONs containing monomer X prompted us to investigate the pyren-1-ylcarbonyl-functionalized 4'-C-piperazinomethyl-DNA monomer Z. As seen in Table 4, incorporation of a single Z monomer induces a remarkable stabilization of duplexes formed with DNA complements

(ΔT_m values of +7.0 and +9.0 °C) but a significant destabilization of the duplexes formed with RNA complements (ΔT_m values of -8.0 and -4.0 °C). Incorporation of two Z monomers substantiates this trend, although the increase in thermal stability per modification towards DNA is significantly lower than that observed for the examples with incorporation of only one Z monomer. Notably, incorporation of three Z monomers leads to a small increase in the thermal stability of the duplex with DNA but no hybridization (above 10 °C) with the RNA complement. These results strongly suggest the ability to engineer the relative thermal stability of short DNA:DNA and DNA:RNA duplexes by incorporation of one or a few 4'-C-(pyren-1-ylcarbonyl)piperazinomethyl monomer(s). The generality of this concept needs to be further studied, but it is encouraging that it is operational when monomer Z is neighboured both by two purine nucleotides and by two pyrimidine nucleotides. The ability to discriminate mis-matched complements as efficiently as the reference ON1 was confirmed for the singly-modified ONs (Table 4). Similar results from denaturation experiments for the 13-mers ON20 and ON21 were obtained (Table 4). Likewise, the pyren-1-ylbutanoyl monomer W (Table 5, ON22-ON25) induced increased T_m values towards DNA complements and reduced T_m values towards RNA complements.

Recognition of double stranded DNA by "dual strand invasion"

The DNA-selectivity induced by monomer Z was explored for targeting double-stranded DNA (dsDNA). Currently, sequence-specific recognition of dsDNA by oligonucleotide analogues is hampered by target sequence limitations and the requirement of unnatural salt concentrations. The absence of cross-hybridization between "ON7" (= ON14 in Table 4 and not ON7 in Table 2), containing three Z monomers, and its RNA complement, suggests the use of "ON7" (= ON14 in Table 4 and not ON7 in Table 2) + RNA as a reagent mixture for recognition of the two complementary segments of a dsDNA duplex (Fig. 2).

Table 2. T_m values (°C) towards DNA/RNA complements.^a

Sequence		110 mM Na ⁺		10 mM Na ⁺	
		DNA	RNA	DNA	RNA
		T_m	T_m	T_m	T_m
5'-d(GTGTTTTGC)	(ON4)	32	32	16	17
5'-d(GT ^L GTT ^L TT ^L GC)	(ON5)	43	55	24	40
5'-d(GTGXTXGC)	(ON6)	35	30	22	17
5'-d(GT ^L GXT ^L XT ^L GC)	(ON7)	47	55	34	42
5'-d(GTGATATGC)	(ON1)	30	26	15	10
5'-d(GT ^L GAXAT ^L GC)	(ON8)	37	42	26	26

^aMelting temperatures [T_m values (ΔT_m values are calculated relative to the T_m value of the reference ON (here **ON4/ON5**)] measured as the maximum of the first derivative of the melting curve (A_{260} vs. temperature; 10 °C to 80 °C with an increase of 1 °C/min) recorded in medium salt buffer [~ 110 mM Na^+] (10 mM sodium phosphate, 100 mM sodium chloride, pH 7.0) and low salt buffer [~ 10 mM Na^+] (10 mM sodium phosphate, pH 7.0) using 1 μM concentrations of the two complementary strands.

We used the duplex **ON1**:DNA as a dsDNA target model and the changes in fluorescence emission of "**ON7**" ("**ON7**" denotes for the rest of this paragraph **ON14** in Table 4 and *not* **ON7** In Table 2) upon hybridization to monitor the processes in solution (Fig. 2; "**ON7**" was present in ca. 2/3 molar ratio to the target). An excimer band at 430-520 nm is seen in the fluorescence emission spectrum of single stranded "**ON7**", which can be explained by the flexibility of the single stranded "**ON7**" allowing the pyrene units to form pyrene-pyrene pairs. No excimer band is observed for the mixture of "**ON7**" and DNA indicating formation of a rigid duplex structure. However, for the mixture of "**ON7**" and RNA, an excimer band is evident and the fluorescence emission spectrum resembles the spectrum of "**ON7**" alone. This corroborates the inability of "**ON7**" and RNA to form a duplex above 10 °C at the applied conditions. The strand invasion experiments were performed at 10 °C at medium salt conditions (110 mM Na^+). First was "**ON7**" added to the preformed target duplex **ON1**:DNA. The strong excimer band observed throughout the experiment (24 h) revealed the absence of significant duplex invasion. However, addition of a mixture of "**ON7**" and RNA to the preformed duplex **ON1**:DNA induced efficient duplex invasion as shown by the absence of an excimer band in the spectrum recorded of this mixture. The importance of addition also of the RNA strand strongly indicates this strand to partake in duplex formation with the **ON1** strand of the original DNA duplex during strand invasion, and the process to involve what can be called "dual strand invasion" (Fig. 2).

Table 3. T_m values (°C) in mis-match experiments.^a

Sequence	110 mM Na^+			
	3'-d(CACTZTACG)			
	Z = A	Z = C	Z = G	Z = T
5'-d(GTGATATGC) (ON1)	28	11	19	12
5'-d(GTGAXATGC) (ON2)	31	19	21	no T_m

^aMelting temperatures [T_m values] measured as the maximum of the first derivative of the melting curve (A_{260} vs. temperature; 10 °C to 80 °C with an increase of 1 °C/min) recorded

in medium salt buffer [~ 110 mM Na⁺] (10 mM sodium phosphate, 100 mM sodium chloride, pH 7.0) using 1 μ M concentrations of the two strands.

Treatment and Therapy

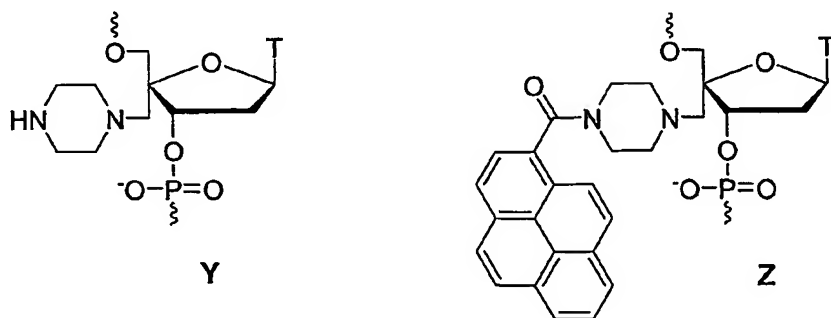
- 5 The preparation of the nucleotide derivatives of the invention has as one purpose their use as a medicament. Correspondingly, the invention is directed to the use of a nucleotide derivative as defined herein for the preparation of a medicament for the treatment of a disease or disorder selected from cancer; diseases caused by viral infections, such as AIDS; influenza, angiogenesis; atherosclerosis, psoriasis, diabetic retinopathy, rheumatoid
- 10 arthritis, asthma, warts, allergic dermatitis and Kaposi sarcoma.

Table 4 ONs synthesized and thermal denaturation studies^a

		DNA target	MM-DNA	RNA target
		$T_m/^\circ\text{C}$	$T_m/^\circ\text{C}$	$T_m/^\circ\text{C}$
ON1	5'-GTGATATGC	29 (ref)	13/18/18	28 (ref)
ON9	5'-GTGAYATGC	31	14/23/18	28
ON10	5'-TGGAZATGC	36	19/29/23	20
ON11	5'-GCAYAYCAC	34		26
ON12	5'-GCAZAZCAC	37		20
ON13	5'-GYGAYAYGC	34		26
ON14	5'-GZGAZAZGC	31		<10
ON15	5'-GTGTTTTGC	32 (ref)		32(ref)
ON16	5'-GTGTZTTGC	41	19/25/24	28
ON17	5'-GTGZTGTGC	34		21
ON18	5'-GZGTTTZGC	34		20
ON19	5'-AAATGATGGCTGC	50		47
ON20	5'-AAATGAZGGCTGC	56		45
ON21	5'-AAAZGAZGGCZGC	59		31

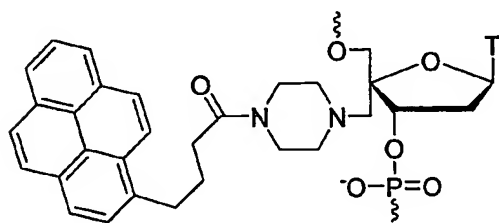
^a Thermal denaturation temperatures [T_m values] measured as the maximum of the first derivative of the melting curve (A_{260} vs. temperature; 10 $^\circ\text{C}$ to 80 $^\circ\text{C}$ with an increase of 1 $^\circ\text{C}/\text{min}$) recorded in medium salt buffer (10 mM sodium phosphate, 100 mM sodium chloride, pH 7.0) using ca. 1 μ M concentrations of the two complementary strands. A, C, G and T are standard DNA monomers. In the column "MM-DNA" are listed T_m values recorded for mis-matched DNA target strands containing a single mis-matched nucleotide; the values are listed in the following order (indicating the mis-matched nucleotide in the central position of the target DNA): C/G/T.

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**Table 5** ONs synthesized and thermal denaturation studies^a

		DNA target	RNA target
		$T_m/^{\circ}\text{C}$	$T_m/^{\circ}\text{C}$
ON1	5'-GTGATATGC	29 (ref)	28 (ref)
ON22	5'-GTGAWATGC	37	28
ON23	5'-GCAWAWCAC	39	23
ON24	5'-GWGAWAWGC	40	<10
ON15	5'-GTGTTTGC	32 (ref)	32 (ref)
ON25	5'-GTGTWTTGC	41	30

^a Thermal denaturation temperatures [T_m values] measured as the maximum of the first derivative of the melting curve (A_{260} vs. temperature; 10 °C to 80 °C with an increase of 1 °C/min) recorded in medium salt buffer (10 mM sodium phosphate, 100 mM sodium chloride, pH 7.0) using ca. 1 μM concentrations of the two complementary strands. A, C, G and T are standard DNA monomers.

**W**

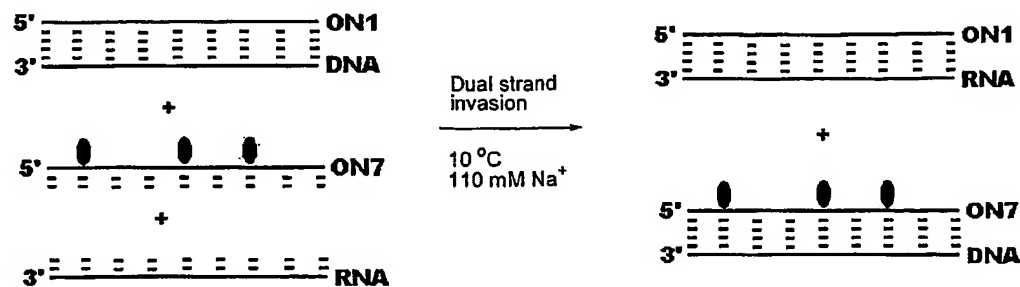


Figure 2. "Dual strand invasion" of dsDNA (**ON1**:DNA duplex). Fluorescence emission spectra of "**ON7**",DNA (15 min), "**ON7**",RNA (15 min), "**ON7**" alone (15 min), **ON1**,DNA after addition of "**ON7**" (24 h), and **ON1**,DNA after addition of "**ON7**", RNA (60 min, see schematic drawing); in parentheses is shown for each spectrum the elapsed time after mixing at which the spectrum was recorded. "**ON7**" was used in ca. 0.15 μ M concentration ($\sim 2/3$ molar ratio to the target); see Table 1 for buffer used. "**ON7**" ("**ON7**" equals **ON14** displayed in Table 4 and *not* **ON7** displayed in Table 2).

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EXAMPLES

Synthesis

Synthesis of 3'-O-(tert-butyldimethylsilyl)-5'-O-(4,4'-dimethoxytrityl)-4'-C-((4-methylpiperazino)methyl)thymidine (2). Nucleoside **1**¹⁷ (500 mg, 0.72 mmol) was dissolved in a mixture of anhydrous dichloromethane (20 ml) and anhydrous pyridine (5 ml). The mixture was cooled to 0 °C and trifluoroacetic anhydride (0.2 ml, 1.4 mmol) was added slowly. The mixture was stirred for 2 hours at 0 °C and was allowed to reach rt. After stirring for 2 hours at rt, the mixture was diluted with dichloromethane (75 ml) and washed with 3x50 ml sat. aq. NaHCO₃. The organic phase was dried (Na₂SO₄) and evaporated to dryness under reduced pressure. The residue was dissolved in a mixture of anhydrous THF (20 ml) and 4-(methyl)piperazine (0.1 ml, 9.0 mmol). The mixture was stirred at rt. for 6 days whereupon the mixture was evaporated to dryness and the residue purified by column chromatography using 0.5-4.0% (v/v) of half sat. methanolic ammonia in dichloromethane furnishing nucleoside **2** as an off-white solid material after pooling and evaporation of the fractions containing **2** (316 mg, 40%). ¹³C-NMR (CDCl₃): δ 163.79, 158.65, 150.21, 144.36, 135.63, 135.49, 130.18, 126.28, 127.86, 127.07, 113.13, 110.72, 89.67, 86.68, 84.10, 71.72, 64.00, 58.68, 55.54, 55.23, 54.67, 45.97, 41.19, 25.69, 17.92, 11.72, -4.57, -5.08.

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Synthesis of 5'-O-(4,4'-dimethoxytrityl)-4'-C-((4-methylpiperazino)methyl)thymidine (3). Nucleoside **2** (150 mg, 0.19 mmol) was dissolved in anhydrous THF (10 ml) and a 1 M solution of tetrabutylammonium fluoride in THF (0.25 ml, 0.25 mmol) was added. The mixture was stirred for 4 hours and was then evaporated to dryness under reduced pressure. The residue was purified by dry column vacuum chromatography using 0-13% methanol in ethylacetate (v/v) as the eluent

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affording nucleoside **3** as a white solid material after pooling and evaporation of the fractions containing **3** (98 mg, 76%). ^{13}C -NMR ($(\text{CD}_3)_2\text{SO}$): δ 163.6, 158.1, 150.3, 149.6, 144.8, 136.1, 135.7, 135.4, 135.3, 129.8, 129.8, 127.9, 127.7, 126.7, 123.9, 113.2, 109.5, 88.2, 86.0, 83.3, 72.1, 65.2, 58.7, 55.0, 54.0, 45.8, 11.7.

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Synthesis of 3'-O-(2-Cyanoethoxy(diisopropylamino)phosphino)-5'-O-(4,4'-dimethoxytrityl)-4'-C-(4-methylpiperazino)methylthymidine (4). Nucleoside **3** (200 mg, 0.30 mmol) was dissolved in a mixture of anhydrous dichloromethane (5 ml) and *N,N*-diisopropylethylamine (0.15 ml, 0.86 mmol), and 2-cyanoethyl(*N,N*-diisopropyl)phosphoramidochloridite (0.08 ml, 0.40 mmol) was added. After stirring for 24 hours at rt, the mixture was diluted with dichloromethane (50 ml) and washed with 3x50 ml sat. aq. NaHCO_3 . The organic phase was dried (Na_2SO_4) and evaporated to dryness under reduced pressure. The residue was purified using column chromatography using a solution of 0.5% triethylamine in 0-5% methanol in dichloromethane (v/v/v) affording amidite **4** as a white solid material after pooling and evaporation of the fractions containing **4** (164 mg, 63%). ^{31}P -NMR ($(\text{CD}_3)_2\text{SO}$): δ 154.8, 154.6.

Synthesis of 3'-O-(tert-Butyldimethylsilyl)-5'-O-(4,4'-Dimethoxytrityl)-4'-C-piperazino-methylthymidine (5). Nucleoside **1** (2.0g, 2.90 mmol) was dissolved in a mixture of anhydrous dichloromethane (16 cm^3) and anhydrous pyridine (4 cm^3). The mixture was cooled to 0 °C and trifluoromethanesulfonic anhydride (0.60 cm^3 , 3.63 mmol) was added slowly under stirring. After 2 hours, the mixture was diluted with dichloromethane (100 cm^3) and washed with saturated aqueous solution of NaHCO_3 (3 x 50 cm^3). The organic phase was dried (Na_2SO_4) and evaporated to dryness under reduced pressure. The residue was dissolved in anhydrous THF (50 cm^3) and piperazine (700 mg, 8.13 mmol) was added. The mixture was heated to 50 °C and stirred for 20 hours, the cooled to room temperature and subsequently evaporated to dryness under reduced pressure. The residue was purified by silica gel column chromatography using 50% saturated methanolic ammonia in dichloromethane (1-6% (v/v) of 50% saturated methanolic ammonia (v/v)) as eluent yielding nucleoside **5** (1.22 g, 1.59 mmol, 54%) as solid white material. ^1H NMR (CDCl_3) δ 7.52 (d, 1H, $J=1.0\text{Hz}$), 7.42-7.44 (m, 2H), 7.23-7.33 (m, 7H), 6.85 (s, 2H), 6.82 (s, 2H), 6.34 (dd, 1H, $J=5.2, 1.6$), 4.70 (t, 1H, $J=6.97$), 3.79 (s, 3H), 3.22 (q, 2H, $J=10.08, 6.32$), 2.82-2.83 (m, 4H), 2.33-2.59 (m, 8H), 2.00-2.08 (m, 1H), 1.41 (s, 3H), 0.84 (s, 9H), 0.37, -0.03 (2 x s, 2 x 3H). ^{13}C NMR (CDCl_3) δ 164.02, 158.61, 150.41, 144.35, 135.61, 135.50, 130.13, 130.10, 128.27, 127.82, 127.03, 113.09, 110.75, 89.55, 86.65, 83.99, 71.85, 64.01, 59.46, 56.00, 55.21, 46.32, 41.08, 25.67, 17.90, 11.75, -4.58, -5.11. MALDI-HRMS m/z 779.38210 ($[\text{M} + \text{Na}]^+$, $\text{C}_{42}\text{H}_{56}\text{N}_4\text{O}_7\text{Si}\cdot\text{Na}^+$); calc. 779.38105.

Synthesis of 3'-O-tert-butylidimethylsilyl-5'-O-(4,4'-dimethoxytrityl)-4'-C-(N-(fluorenylmethoxycarbonyl)piperazino)methylthymidine (6a). Nucleoside **2** (1.22 g, 1.58 mmol) was dissolved in anhydrous pyridine (30 cm³) at rt and 9-Fluorenylmethyl chloroformate (563 mg, 2.18 mmol) was added under stirring. After 1 hour, the mixture was evaporated to dryness under reduced pressure. The residue was coevaporated with anhydrous acetonitrile and was purified by silica gel column chromatography using pyridine/DCM (0.5% pyridine in dichloromethane (v/v)) as eluent yielding nucleoside **6a** (1.51 g, 1.55 mmol, 98%) as a white solid material. ¹H NMR (CDCl₃) δ 8.91 (s, 1H), 8.66-8.75 (m, 1H), 7.81, 7.79 (2xs, 2H), 7.29-7.63 (m, 16H), 6.89, 6.86 (2xs, 4H), 6.39 (t, 1H, J=6.08), 4.75 (t, 1H, J=6.55), 4.46 (d, 1H, J=1.38), 4.44 (s, 1H), 4.27 (t, 1H, J=6.86), 3.83 (s, 3H), 3.29-3.45 (m, 6H), 2.45-2.67 (m, 7H), 2.03-2.12 (m, 2H), 1.48 (s, 3H), 0.90 (s, 9H), 0.10 (s, 3H), 0.04 (s, 3H). ¹³C NMR (CDCl₃) δ 163.72, 158.67, 155.09, 150.25, 149.72, 144.25, 144.01, 143.98, 141.27, 135.94, 135.54, 135.45, 135.42, 130.11, 130.06, 128.25, 127.87, 127.60, 127.12, 126.99, 124.95, 123.68, 119.92, 113.13, 110.93, 89.41, 86.77, 83.98, 72.19, 67.18, 64.10, 58.99, 55.23, 54.33, 47.32, 44.07, 41.00, 25.69, 17.93, 11.77, -4.57, -5.10. MALDI-HRMS *m/z* 1001.45190 ([M + Na]⁺, C₅₇H₆₆N₄O₉Si·Na⁺); calc. 1001.44913.

Synthesis of 5'-O-(4,4'-dimethoxytrityl)-4'-C-(N-(fluorenylmethoxycarbonyl)piperazino)-methylthymidine (7a). Nucleoside **6a** (1.51 g, 1.55 mmol) was dissolved in anhydrous THF (80 cm³) and to the stirred mixture at rt was added a mixture of triethylamine tris-hydrofluoride (4.5 cm³, 27.6 mmol) and pyridine hydrochloride (2.00 g, 17.31 mmol). After 7 hours, the mixture was diluted with dichloromethane (250 cm³) and washed with a saturated aqueous solution of NaHCO₃ (3 x 100 cm³). The organic phase was dried (Na₂SO₄) and evaporated to dryness under reduced pressure. The residue was purified by silica gel column chromatography using pyridine/dichloromethane (0.5% pyridine in dichloromethane (v/v)) as eluent yielding nucleoside **7a** (941 mg, 1.09 mmol, 70%) as a white solid material. ¹H NMR (CDCl₃) δ 9.71 (s, 1H), 8.61-8.61 (m, 3H), 7.22-7.75 (m, 23H), 6.86 (s, 2H), 6.83 (s, 2H), 6.46 (t, 1H, J = 4.52), 4.65 (d, 1H, 4.39), 4.42 (d, 2H J= 6.41), 4.22 (t, 1H, 6.76), 3.78 (s, 3H), 3.46 (br s, 4H), 3.16 (d, 1H, J = 9.66), 3.07 (d, 1H, J = 9.69), 2.36-2.91 (m, 9H), 1.57 (3H, s). ¹³C NMR (CDCl₃) δ 163.78, 158.70, 154.86, 150.56, 149.66, 143.98, 143.85, 141.22, 135.91, 135.08, 135.06, 134.90, 129.99, 128.03, 127.96, 127.61, 127.16, 126.98, 124.87, 123.65, 119.90, 113.22, 111.13, 87.11, 86.51, 84.25, 75.92, 67.25, 66.82, 60.59, 55.17, 54.51, 47.23, 43.77, 41.20, 11.98. MALDI-HRMS *m/z* 887.36240 ([M + Na]⁺, C₅₁H₅₂N₄O₉·Na⁺); calc. 887.36265.

Synthesis of 3'-O-(2-cyanoethoxy)(diisopropylamino)phosphino-5'-O-(4,4'-dimethoxytrityl)-4'-C-(N-(fluorenylmethoxycarbonyl)piperazino)methylthymidine (8a). Nucleoside **7a** (425 mg, 0.49 mmol) was dissolved in anhydrous dichloromethane (10 cm³) and the stirred mixture at rt was added *N,N*-diisopropylethylamine (0.25 cm³). 2-Cyanoethyl *N,N*-diisopropylphosphoramido-chloridite (0.13 cm³, 0.52 mmol) was added slowly, and after 2 hours the mixture was evaporated to dryness under reduced pressure. The residue was purified by silica gel column chromatography using ethylacetate/*n*-heptane/triethylamine (50-60% EtOAc and 0.5% triethylamine in *n*-heptane (v/v/v)) as eluent yielding amidite **8a** (287 mg, 0.27 mmol, 54%) as a white solid material. ³¹P NMR (DMSO-*d*₆) δ 150.14, 149.81.

Synthesis of 3'-O-*tert*-butyldimethylsilyl-5'-O-(4,4'-dimethoxytrityl)-4'-C-(N-(pyren-1-ylcarbonyl)piperazino)methylthymidine (6b). Nucleoside **5** (300 mg, 0.39 mmol) was dissolved in anhydrous dichloromethane (6 cm³), and to the stirred mixture at rt was added 1-pyrenecarboxylic acid (170 mg, 0.59 mmol) and *N,N*-diethylcarbodiimide (120 mg, 1.22 mmol). After 3 hours, dichloromethane (50 cm³) was added followed by washing using a saturated aqueous solution of NaHCO₃ (3 x 20 cm³). The organic phase was dried (Na₂SO₄) and evaporated to dryness under reduced pressure. The residue was purified twice by silica gel column chromatography using DCM and pyridine (0.5% pyridine in dichloromethane (v/v)) as eluent yielding nucleoside **6b** (288 mg, 0.29 mmol, 75%) as a white solid material. ¹³C NMR (CDCl₃) δ 169.679, 163.63, 158.67, 150.20, 150.13, 149.74, 144.23, 135.49, 135.39, 135.34, 131.57, 131.16, 130.95, 130.11, 130.05, 128.74, 128.65, 128.21, 128.14, 127.88, 127.38, 127.12, 126.28, 125.68, 125.57, 124.71, 124.62, 124.54, 124.46, 123.92, 123.79, 123.68, 113.13, 110.94, 110.87, 89.37, 86.79, 83.98, 83.91, 72.29, 72.11, 64.20, 64.10, 58.88, 58.80, 55.20, 54.89, 54.78, 54.69, 47.58, 42.25, 41.08, 25.67, 17.92, 11.70, -4.59, -5.12. MALDI-HRMS *m/z* 1007.4420 ([M + Na]⁺, C₅₉H₆₄N₄O₈Si·Na⁺); calc. 1007.43856.

5'-O-(4,4'-Dimethoxytrityl)-4'-C-(N-(pyren-1-ylcarbonyl)piperazino)methylthymidine (7b). Nucleoside **6b** (275 mg, 0.282 mmol) was dissolved in anhydrous THF (7 cm³) under stirring at rt, and tetrabutylammoniumfluoride (1M solution, 0.45 cm³, 0.45 mmol) was added. After 16 hours, ethylacetate (50 cm³) was added, whereupon washing was performed using a saturated aqueous solution of NaHCO₃ (3 x 30 cm³). The organic phase was dried (Na₂SO₄) and evaporated to dryness under reduced pressure. The residue was purified by silica gel column chromatography using pyridine/DCM (0.5% pyridine in dichloromethane (v/v)) as eluent yielding nucleoside **7b** (200 mg, 0.222 mmol, 81%) as a white solid material. ¹³C NMR (CDCl₃) 169.7, 163.6, 158.7, 150.4, 149.6, 144.0, 136.0, 135.1, 135.0, 134.9, 131.9, 131.1, 130.7, 130.3, 130.0, 128.8, 128.2, 128.0, 128.0, 127.4, 127.1, 127.1,

126.3, 125.8, 125.6, 124.4, 123.9, 113.2, 111.1, 87.1, 86.6, 84.1, 76.0, 66.8, 60.5, 55.2, 54.9, 47.3, 47.2, 41.9, 41.1, 11.9. MALDI-HRMS m/z 893.35200 ($[M + Na]^+$, $C_{53}H_{50}N_4O_8 \cdot Na^+$); calc. 893.35208.

5 **3'-O-(2-Cyanoethoxy)(diisopropylamino)phosphino-5'-O-(4,4'-dimethoxytrityl)-4'-C-(N-(pyren-1-ylcarbonyl)piperazino)methylthymidine (8b)**. Nucleoside **7b** (200 mg, 0.22 mmol) was coevaporated with acetonitrile ($2 \times 7 \text{ cm}^3$) and dissolved in anhydrous dichloromethane (7 cm^3) under stirring at rt. *N,N*-diisopropylethylamine (0.15 cm^3) was added followed by slow addition of 2-cyanoethyl *N,N*-diisopropylphosphoramidochloridite (0.10 cm^3 , 0.40 mmol). After 5 hours, the mixture was evaporated to dryness under reduced pressure. The residue was purified by silica gel column chromatography using ethylacetate/*n*-heptane/triethylamine (50-60% EtOAc and 0.5% triethylamine in *n*-heptane (v/v/v)) as eluent yielding amidite **8b** (163 mg, 0.15 mmol, 67%) as a white solid material. ^{31}P NMR ($\text{DMSO}-d_6$) δ 150.08, 149.85.

15 **3-O-tert-Butyldimethylsilyl-5'-O-(4,4'-dimethoxytrityl)-4'-C-(N-(4-(pyren-1-yl)butanoyl)piperazino)methylthymidine (6c)**. Nucleoside **5** (300 mg, 0.39 mmol) was dissolved in anhydrous dichloromethane (6 cm^3) under stirring at rt and 1-pyrenebutanoic acid (170 mg, 0.59 mmol) was added together with *N,N*-diethylcarbodiimide (120 mg, 1.22 mmol). After 1 hour, dichloromethane (50 cm^3) was added and washing was performed using a saturated aqueous solution of NaHCO_3 ($3 \times 30 \text{ cm}^3$). The organic phase was dried (Na_2SO_4) and evaporated to dryness under reduced pressure. The residue was purified by silica gel column chromatography using methanol in a mixture of dichloromethane and pyridine (0-1.5% MeOH, 0.5% pyridine in dichloromethane (v/v/v)) as eluent yielding nucleoside **6c** (302 mg, 0.29 mmol, 74%) as a white solid material. ^1H NMR (CDCl_3) 8.96 (s, 1H), 8.42 (d, 1H, $J = 9.09$), 8.23-7.91 (m, 9H), 7.53-7.30 (m, 12H), 6.89 (2H, s), 6.87 (s, 2H), 6.39 (t, 1H, $J = 6.07$), 4.76 (t, 1H, $J = 6.62$), 3.83 (s, 6H), 3.63 (br s, 2H), 3.48 (t, 2H, $J = 7.42$), 3.37-3.24 (m, 4H), 2.64-2.02 (m, 13H), 1.49 (s, 3H), 0.91 (s, 9H), 0.11 (s, 3H), 0.05 (s, 3H). ^{13}C NMR (CDCl_3) δ 170.99, 163.75, 158.66, 150.26, 144.22, 136.10, 135.52, 135.45, 135.39, 131.35, 130.87, 130.09, 130.05, 129.83, 128.79, 128.23, 127.87, 127.79, 127.44, 127.32, 127.30, 127.11, 127.07, 126.59, 125.75, 125.01, 124.94, 124.78, 124.74, 124.68, 123.48, 113.12, 110.94, 89.41, 86.75, 83.92, 72.12, 64.06, 58.81, 55.20, 54.63, 54.43, 45.62, 41.88, 41.01, 32.81, 32.38, 26.92, 25.67, 17.91, 11.76, -4.60, -5.11. MALDI-HRMS m/z 1049.49020 ($[M + Na]^+$, $\text{C}_{62}\text{H}_{70}\text{N}_4\text{O}_8\text{Si} \cdot \text{Na}^+$); calc. 1049.4851.

5'-O-(4,4'-Dimethoxytrityl)-4'-C-(N-(4-(pyren-1-yl)butanoyl)piperazino)methylthymidine (7c). Nucleoside **6c** (290 mg, 0.282 mmol) was dissolved in anhydrous THF (7 cm^3) under stirring at rt and tetrabutylammoniumfluoride

(1M solution, 0.45 cm³, 0.45 mmol) was added. After 16 hours, ethylacetate (50 cm³) was added and washed was performed using a saturated aqueous solution of NaHCO₃ (3 x 30 cm³). The organic phase was dried (Na₂SO₄) and evaporated to dryness under reduced pressure. The residue was purified by silica gel column chromatography using

5 pyridine/dichloromethane (0.5% pyridine in dichloromethane (v/v)) as eluent yielding nucleoside **7c** (207 mg, 0.222 mmol, 78%) as a white solid material. ¹H NMR (CDCl₃) 9.23 (br s, 1H), 8.51 (d, 1H, *J*=4.11), 8.18 (d, 1H, *J*=9.34), 8.02-7.80 (m, 8H), 7.71 (d, 1H, *J*=7.77), 7.28-7.10 (m, 12H), 6.23 (s, 2H), 6.70 (s, 2H), 6.34-6.30 (1H), 4.52 (d, 1H, *J*=4.56), 3.65 (s, 6H), 3.60-3.25 (m, 5H), 2.98 (d, 1H, *J*=9.79), 2.87 (d, 1H, *J*=9.60),

10 2.69-2.05 (m, 13H), 1.43 (s, 3H). ¹³C NMR (CDCl₃) δ 170.97, 163.61, 158.71, 150.64, 149.67, 143.95, 135.98, 135.93, 135.12, 135.04, 134.87, 131.31, 130.81, 130.01, 129.80, 128.74, 128.03, 127.94, 127.41, 127.28, 127.18, 126.57, 125.73, 124.97, 124.89, 124.77, 124.72, 124.65, 123.67, 123.42, 113.23, 111.25, 87.14, 86.46, 84.08, 76.04, 66.85, 60.50, 55.18, 54.79, 54.60, 45.27, 41.56, 41.09, 32.70, 32.21, 26.80,

15 11.95. MALDI-HRMS *m/z* 935.29890 ([*M* + Na]⁺, C₅₆H₅₆N₄O₈·Na⁺); calc. 935.39903.

3'-O-(2-Cyanoethoxy)(diisopropylamino)phosphino-5'-O-(4,4'-dimethoxytrityl)-4'-C-(N-(4-(pyren-1-yl)butanoyl)piperazino)methylthymidine (8c). Nucleoside **7c** (200 mg, 0.22 mmol) was dissolved in anhydrous dichloromethane (7 cm³) under stirring

20 at rt whereupon *N,N*-diisopropylethylamine (0.15 cm³) was added. 2-Cyanoethyl *N,N*-diisopropylphosphoramidochloridite (0.10 cm³, 0.40 mmol) was added slowly, and after 2 hours the mixture was evaporated to dryness under reduced pressure. The residue was purified by silica gel column chromatography using ethylacetate/*n*-heptane/triethylamine (50-60% EtOAc and 0.5% triethylamine in *n*-heptane (v/v/v)) as eluent yielding amidite

25 **8c** (157 mg, 0.14 mmol, 64%) as a white solid material. ³¹P NMR (DMSO-*d*₆) δ 149.89, 149.68.

General procedure for on-column oligonucleotide conjugation. The oligonucleotide (JW789: 5'-GTGAXATGC; JW878: 5'-GTGATATGCX) was synthesized using ordinary

30 phosphoramidite conditions without final detritylation step in order to leave the 5'-end DMT-protected. The oligonucleotide was synthesized in a 1 μmol scale using polystyrene as the solid support. The supports were transferred from the reaction vessels and to an eppendorf tube and treated with 20% piperidine in DMF (1 cm³) for 20 min. The supernatant was aspirated with a syringe, using a small needle, and the polystyrene was

35 washed with acetonitrile (4 x 1 cm³). The polystyrene was suspended in anhydrous DMF (1 cm³) and divided into three approximately even sized portions by transfer into three eppendorf tubes. A mixture of acid to be conjugated (15 μmol), HBTU (3,7 mg, 9.5 μmol), *N,N*-diisopropylethylamine (10 μl, 5.7 μmol) and DMF (1 cm³) was added to the

polystyrene support and vortexed gently for 45 min. The supernatant was aspirated using syringe with a small needle and the polystyrene was washed with DMF ($2 \times 1 \text{ cm}^3$) and MeOH ($2 \times 1 \text{ cm}^3$). The oligonucleotide was released from the solid support by treatment with saturated methanolic ammonia for 24 hours. This treatment also cleaved the

5 protecting groups of the nucleobases. The 5'-end DMT-protected oligonucleotide was then purified by reversed phase HPLC. The DMT group was cleaved off by treatment with 80% AcOH (100 μl) for 20 min, followed by addition of sodium acetate (3M, 50 μl), water (100 μl) and ethanol (600 μl). The solution was cooled to -18°C for 1 hour causing the oligonucleotide to precipitate. The oligonucleotide was then isolated after centrifuging at 5

10 $^\circ\text{C}$ by decanting of the supernatant. The conjugated oligonucleotide was finally desalted using a NAP-column. Example of conjugates made using this procedure (JW789: GTG AXA TGC; JW878: GTG ATA TGC X): **JW878 Asp.** Aspartic acid was coupled in the protected form Fmoc-Asp-(2-phenylisopropylester)-OH (15 μmol , 50 eq. 7.10 mg). Yield 4.9 OD in 1 ml H_2O equal to 0.05 μmol (15%). **JW878 Gly.** Glycine was coupled in the protected form

15 Fmoc-Gly-OH (15 μmol , 50 eq. 4.46 mg). Yield 3.5 OD in 1 ml H_2O equal to 0.035 μmol (11%). **JW878 Trp.** Tryptophan was coupled in the protected form Fmoc-Trp-OH (15 μmol , 50 eq. 6.39 mg). Yield 3.9 OD in 1 ml H_2O equal to 0.04 μmol (12%). **JW878 Phe.** Phenylalanine was coupled in the protected form Fmoc-Phe-OH (15 μmol , 50 eq. 5.81 mg). Yield 1.9 OD in 1 ml H_2O equal to 0.02 μmol (6%). **JW878 Leu.** Leucine was coupled in

20 the protected form Fmoc-Leu-OH (15 μmol , 50 eq. 5.30 mg). Yield 2.5 OD in 1 ml H_2O equal to 0.025 μmol (7.5%). **JW878 His.** Histidine was coupled in the protected form $\text{N}_\alpha, \text{N}_{(\text{im})}$ -di-His-OH (15 μmol , 50 eq. 8.99 mg). Yield 3.2 OD in 1 ml H_2O equal to 0.033 μmol (10%). **JW878 Lys.** Lysine was coupled in the protected form $\text{N}_\alpha, \text{N}_\epsilon$ -di-Fmoc-Lys-OH (15 μmol , 50 eq. 8.86 mg). Yield 4.5 OD in 1 ml H_2O equal to 0.046 μmol (14%). **JW789**

25 **Biotin.** Biotin was coupled (15 μmol , 50 eq. 3.66 mg). Yield 2.6 OD in 1 ml H_2O equal to 0.029 μmol (9%). **JW789 1-pyrenecarboxylic acid.** 1-Pyrenecarboxylic acid was coupled (15 μmol , 50 eq. 3.69 mg). Yield 4.6 OD in 1 ml H_2O equal to 0.051 μmol (15%). **JW789 4-(pyren-yl)butanoic acid.** 4-(Pyren-1-yl)butanoic acid was coupled (15 μmol , 50 eq. 4.332 mg). Yield 3.9 OD in 1 ml H_2O equal to 0.043 μmol (13%).

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All patents, patent applications, and publications cited herein are hereby incorporated by reference. The foregoing written specification is considered to be sufficient to enable one skilled in the art to practice the invention. Indeed, various modifications of the above-described invention which are obvious to those skilled in the field of organic chemistry or

35 related fields are intended to be within the scope of the following claims.

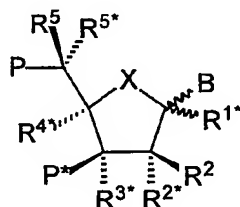
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CLAIMS

- 5 1. A nucleotide derivative which in its 4' and/or 5' position on the sugar moiety is substituted with a group comprising a non-aromatic cyclic group comprising at least one nitrogen atom, said cyclic group optionally being substituted.
2. A nucleoside derivative having the structure (In any configuration):

10



- 15 wherein X is selected from -O-, -S-, -N(R⁶)-, -C(R⁶R^{6*})-;

B is selected from hydrogen, hydroxy, optionally substituted C₁₋₄-alkoxy, optionally substituted C₁₋₄-alkyl, optionally substituted C₁₋₄-acyloxy, nucleobases (preferably a base selected from the group consisting of Adenine, Guanine, Cytosine, Uracil, Thymine and derivatives thereof);

20

P designates the radical position for an internucleoside linkage to a succeeding monomer, or a 5'-terminal group such as OH or a OH protection group (eg. OCEPA, OTs);

- 25 P* designates an internucleoside linkage to a preceding monomer, or a 3'-terminal group such as OH or a OH protection group (eg. OCEPA, OTs);

each of the substituents R^{1*}, R², R^{2*}, R^{3*}, R^{4*}, R⁵, R^{5*}, R⁶, and R^{6*} is independently selected, preferably from hydrogen, C₁₋₁₂-alkyl, C₂₋₁₂-alkenyl, C₂₋₁₂-alkynyl, hydroxy, C₁₋₁₂-alkoxy, C₂₋₁₂-alkenyloxy, mercapto, C₁-C₁₂ alk(en)ylthio, carboxy, C₁₋₁₂-alkoxycarbonyl, C₁₋₁₂-alkylcarbonyl, formyl, aryl, aralkyl, aryloxy-carbonyl, aryloxy, arylthio, aralkoxy, arylcarbonyl, heterocyclyl, heterocyclyl-alkyl, heterocyclioxy-carbonyl, heterocyclyl-oxy, heterocyclyl-carbonyl, amino, mono- and di(C₁₋₆-alkyl)amino, carbamoyl, mono- or di(C₁₋₆-alkyl)-amino-carbonyl, amino-C₁₋₆-alkyl-aminocarbonyl, mono- or di(C₁₋₆-alkyl)amino-C₁₋₆-alkyl-aminocarbonyl, C₁₋₆-alkyl-carbonylamino, carbamido, C₁₋₆-alkanoyloxy, sulphonyloxy, C₁₋₆-alkylsulphonyloxy, nitro, azido, sulphonyl, C₁₋₆-alkylthio, halogen;

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any of said groups above optionally being substituted with one or more substituents, preferably selected from:

C₁-C₆ alkyl, C₁-C₆ alkoxy, aralkyl, aryl, heterocyclyl, acyl, halogen, nitro, hydroxy, amino, CN, N₃, CF₃, NR₂, OH, OR, SH, SR, OR, COOH, COOR, SO₃-R, where R is selected from the group consisting of H, alkyl, aralkyl, aryl, acyl, CF₃-CO;

- 5 and/or where two geminal substituents together may designate oxo, thioxo, imino, or optionally substituted methylene;

carbon chains (such as alkyl, alkenyl) being saturated or unsaturated C₁-C₁₂, preferably C₁-C₆;

10

provided that at least one of the substituents R^{4*} and R^{5*} contains a nitrogen containing non-aromatic heterocyclic ring;

and salts, such as basic salts and acid addition salts thereof.

15

3. The nucleoside derivative of claim 2 wherein R^{1*}, R², R^{3*}, and R⁵ independently are selected from the group consisting of hydrogen, alkyl, substituted alkyl, aralkyl, substituted aralkyl, aryl, and substituted aryl, the substituents being defined as in claim 2.

- 20 4. The nucleoside derivative according to any of claims 2-3, wherein R^{2*} is selected from the group consisting of hydrogen, hydroxy, alkyl, substituted alkyl, aralkyl, substituted aralkyl, aryl, and substituted aryl, the substituents being defined as in claim 2.

- 25 5. The nucleoside derivative according to any of claims 2-4 wherein R^{4*} contains a nitrogen containing non-aromatic heterocyclic ring linked to the sugar moiety of the nucleotide by a group selected from: C₁-C₁₂ alkylene (eg. methylene, ethylene); C₁-C₁₂ alkylene wherein one or more carbon atoms are replaced with a heteroatom (eg. oxymethylene, methyleneoxy, methyleneimino, thiomethylene, methylenethio); C=O (carbonyl); a heteroatom (eg. oxy or thio); N-R⁶ (R⁶ defined as in claim 2); or a
30 combination of two or more of these groups.

- 35 6. The nucleoside derivative according to any of claims 2-4 wherein R^{5*} contains a nitrogen- containing non-aromatic heterocyclic ring linked to the sugar moiety of the nucleotide by a group selected from: C₁-C₁₂ alkylene (eg. methylene, ethylene); C₁-C₁₂ alkylene wherein one or more carbon atoms are replaced with a heteroatom (eg. oxymethylene, methyleneoxy, methyleneimino, thiomethylene, methylenethio); C=O (carbonyl); a heteroatom (eg. oxy or thio); N-R⁶ (R⁶ defined in claim 2) or a combination of two or more of these groups.

- 40 7. The nucleoside derivative of any of claims 2-6, wherein the non-aromatic nitrogen - containing non-aromatic heterocyclic ring in R^{4*} and/or R^{5*} is selected from a ring having 5, 6 or 7 ring members, said ring members being at least one nitrogen, and the rest of the ring members are independently selected from carbon, oxygen, sulphur and nitrogen; said ring containing no or one double bond; and said ring optionally being substituted with a
45 group preferably selected from: C₁-C₆ alkyl, C₁-C₆ alkoxy, aralkyl, aryl, acyl, halogen, nitro,

hydroxy, amino, CN, N₃, CF₃, NR₂, OH, OR, SH, SR, OR, COOH, COOR, SO₃-R, where R is selected from the group consisting of H, alkyl, aralkyl, aryl, acyl, CF₃-CO; etc.

8. The nucleoside derivative according to claim 7 wherein the nitrogen-containing non-
5 aromatic heterocyclic ring is selected from piperazine, piperidine, pyrrolidine or morpholine; any of which is optionally substituted with a substituent R⁶ as defined in claim 2; and being coupled to the linker via a ring carbon or nitrogen atom.

9. A nucleotide derivative according to any of the preceding claims, which is a 4'-C-(4-
10 methylpiperazino)methyl-, a 4'-C-(piperidino)methyl-, a 4'-C-(pyrrolidino)methyl-, or a 4'-C-(piperazino)methyl-derivative.

10. The nucleoside derivative according to any one of claims 7 and 8, wherein the
15 nitrogen-containing non-aromatic heterocyclic ring is optionally substituted with aralkyl or acyl.

11. The nucleoside derivative according to any one of claims 7-8 or 10, wherein the
nitrogen-containing non-aromatic heterocyclic ring is optionally substituted with R⁷-alkyl, R⁷-alkyl-carbonyl, or R⁷-alkyl-thiocarbonyl, R⁷ being an aromatic or non-aromatic, cyclic or
20 heterocyclic ring-system with from 6 to 24 atoms, such as indoliziny, indolyl, isoindolyl, indoliny, benzofuranyl, benzothiophenyl, indazolyl, benzimidazolyl, benzthiazolyl, purinyl, quinoliziny, quinoliny, isoquinoliny, cinnoliny, phtalaziny, quinazoliny, quinoxaliny, naphthyridiny, pteridiny, chromanyl, isochromanyl, carbazolyl, acridiny, phenaziny, phenothiaziny, phenoxaziny, thianthrenyl, indenyl, naphthalenyl, anthracenyl,
25 acenaphthylenyl, fluorenyl, phenathrenyl, biphenylenyl, phenalenyl, tetracenyl, pyrenyl, triphenylenyl, chrysenyl, aceanthrylenyl, acephenanthrylenyl, fluoranthenyl, pleiadenyl, perylenyl, chromenyl, isochromenyl, xanthylenyl, phenanthridiny, benztriazolyl, peridimidiny, phenanthroliny, and oxanthrenyl.

30 12. A nucleotide derivative according to claim 11, which is a 4'-C-(4-(pyren-1-ylcarbonyl)-piperazino)methyl-, 4'-C-(4-(pyren-1-ylbutanoyl)-piperazino)methyl-derivative, 4'-C-(4-(pyren-1-ylcarbonyl)-pyrrolidino)methyl-, 4'-C-(4-(pyren-1-ylbutanoyl)-pyrrolidino)methyl-derivative, 4'-C-(4-(pyren-1-ylcarbonyl)-piperidino)methyl-, or 4'-C-(4-(pyren-1-ylbutanoyl)-piperidino)methyl-derivative.

35 13. An oligonucleotide containing at least one nucleoside derivative of any of the preceding claims.

14. An oligonucleotide containing at least one nucleoside derivative of claims 1-9, which
40 oligonucleotide has a length of 5-50 nucleotide units, preferably 10-30 units, most preferably 12-20 units.

15. An oligonucleotide according to any one of claims 13-14 which is labelled, such as
45 radio-labelled.

16. A polynucleotide containing at least one nucleotide or oligonucleotide according to any of the preceding claims.
17. A polynucleotide or oligonucleotide according to any of the preceding claims, wherein
 5 any internucleoside linkage (the divalent linker group that forms the covalent linking of two adjacent nucleosides, between the 3' carbon atom on the first nucleoside and the 5' carbon atom on the second nucleoside (said nucleosides optionally being 3',5' dideoxy)) is selected from linkages consisting of 2 to 4, preferably 3, groups/atoms selected from -CH₂-
 , -O-, -S-, -NR^H-, >C=O, >C=NR^H, >C=S, -Si(R'')₂-, -SO-, -S(O)₂-, -P(O)₂-, -P(O,S)-, -
 10 P(S)₂-, -PO(R'')-, -PO(OCH₃)-, and -PO(NHR^H)-, where R^H and R'' has the same meaning as R⁶ in claim 2, or are preferably selected from hydrogen, C₁₋₆-alkyl and phenyl.
18. An oligonucleotide or polynucleotide according to claim 17, wherein any internucleoside linkage is selected from -CH₂-CH₂-CH₂-, -CH₂-CO-CH₂-, -CH₂-CHOH-CH₂-, -O-CH₂-O-,
 15 -O-CH₂-CH₂-, -O-CH₂-CH=, -CH₂-CH₂-O-, -NR^H-CH₂-CH₂-, -CH₂-CH₂-NR^H-, -CH₂-NR^H-CH₂-,
 -O-CH₂-CH₂-NR^H-, -NR^H-CO-O-, -NR^H-CO-NR^H-, -NR^H-CS-NR^H-, -NR^H-C(=NR^H)-NR^H-, -NR^H-
 CO-CH₂-NR^H-, -O-CO-O-, -O-CO-CH₂-O-, -O-CH₂-CO-O-, -CH₂-CO-NR^H-, -O-CO-NR^H-, -NR^H-
 CO-CH₂-, -O-CH₂-CO-NR^H-, -O-CH₂-CH₂-NR^H-, -CH=N-O-, -CH₂-NR^H-O-, -CH₂-O-N=, -CH₂-
 O-NR^H-, -CO-NR^H-CH₂-, -CH₂-NR^H-O-, -CH₂-NR^H-CO-, -O-NR^H-CH₂-, -O-NR^H-, -O-CH₂-S-,
 20 -S-CH₂-O-, -CH₂-CH₂-S-, -O-CH₂-CH₂-S-, -S-CH₂-CH=, -S-CH₂-CH₂-, -S-CH₂-CH₂-O-, -S-
 CH₂-CH₂-S-, -CH₂-S-CH₂-, -CH₂-SO-CH₂-, -CH₂-SO₂-CH₂-, -O-SO-O-, -O-S(O)₂-O-, -O-
 S(O)₂-CH₂-, -O-S(O)₂-NR^H-, -NR^H-S(O)₂-CH₂-, -O-S(O)₂-CH₂-, -O-P(O)₂-O-, -O-P(O,S)-O-, -
 O-P(S)₂-O-, -S-P(O)₂-O-, -S-P(O,S)-O-, -S-P(S)₂-O-, -O-P(O)₂-S-, -O-P(O,S)-S-, -O-
 P(S)₂-S-, -S-P(O)₂-S-, -S-P(O,S)-S-, -S-P(S)₂-S-, -O-PO(R'')-O-, -O-PO(OCH₃)-O-,
 25 -O-PO(BH₃)-O-, -O-PO(NHR^N)-O-, -O-P(O)₂-NR^H-, -NR^H-P(O)₂-O-, -O-P(O,NR^H)-O-, and -O-
 Si(R'')₂-O-, where R^H and R'' has the same meaning as R⁶ in claim 2.
19. An oligonucleotide or polynucleotide according to claim 17-18, wherein any
 Internucleoside linkage is selected from -CH₂-CO-NR^H-, -CH₂-NR^H-O-, -S-CH₂-O-, -O-P(O)₂-
 30 O-, -O-P(O,S)-O-, -O-P(S)₂-O-, -NR^H-P(O)₂-O-, -O-P(O,NR^H)-O-, -O-PO(R'')-O-,
 -O-PO(CH₃)-O-, and -O-PO(NHR^N)-O-, where R^H is selected from hydrogen and C₁₋₄-alkyl,
 and R'' is selected from C₁₋₆-alkyl and phenyl.
20. An oligonucleotide or polynucleotide according to any of the preceding claims, which
 35 has enzymatic activity (e.g. being a DNAzyme), or is able to bind to a polypeptide (such as an enzyme) or to a polynucleotide (such as RNA (eg. siRNA) or DNA).
21. An oligonucleotide according to any of the preceding claims, covalently conjugated to an amino acid sequence, such as an enzyme or a active part thereof.
- 40 22. A oligonucleotide according to any of the preceding claims, covalently conjugated to a amino acid sequence or another moiety for enhanced cell membrane penetration and or intra- or extracellular localization.

23. A composition, such as a pharmaceutical composition, comprising at least one nucleotide and/or oligonucleotide and/or polynucleotide according to any of the preceding claims.
- 5 24. A pharmaceutical composition comprising at least one nucleotide and/or oligonucleotide and/or polynucleotide according to any of the preceding claims, and a pharmaceutically acceptable carrier.
- 10 25. A method for producing a compound of formula I as defined in claim 2, which comprises reacting a compound of formula I, wherein the at least one of the substituents R⁴* and R⁵* contains a hydroxy group, with a optionally substituted non-aromatic ring containing at least one nitrogen atom a ring member, the substituents being defined as in claim 2.
- 15 26. A nucleotide derivative according to 2 for use as a medicament.
27. Use of a nucleotide derivative according to claim 2 for the preparation of a medicament for the treatment of a disease or disorder selected from cancer; diseases caused by viral infections, such as AIDS; Influenza, angiogenesis; arteriosclerosis, psoriasis, diabetic
- 20 retinopathy, rheumatoid arthritis, asthma, warts, allergic dermatitis and Karposis sarcoma.

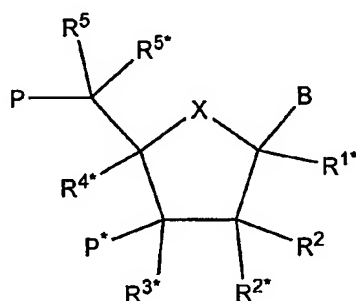
AMENDED CLAIMS

[Received by the International Bureau on 01 November 2004 (01.11.2004)
original claim 2 amended]

CLAIMS

5 1. A nucleotide derivative which in its 4' and/or 5' position on the sugar moiety is substituted with a group comprising a non-aromatic cyclic group comprising at least one nitrogen atom, said cyclic group optionally being substituted.

2. A nucleoside derivative having the structure (in any configuration):
10



15 wherein X is selected from -O-, -S-, -N(R⁶)-, -C(R⁶R^{6*})-;

B is selected from hydrogen, hydroxy, optionally substituted C₁₋₄-alkoxy, optionally substituted C₁₋₄-alkyl, optionally substituted C₁₋₄-acyloxy, nucleobases (preferably a base selected from the group consisting of Adenine, Guanine, Cytosine, Uracil, Thymine and
20 derivatives thereof);

P designates the radical position for an internucleoside linkage to a succeeding monomer, or a 5'-terminal group such as OH or a OH protection group (eg. OCEPA, OTs);

25 P* designates an internucleoside linkage to a preceding monomer, or a 3'-terminal group such as OH or a OH protection group (eg. OCEPA, OTs);

each of the substituents R^{1*}, R², R^{2*}, R^{3*}, R^{4*}, R⁵, R^{5*}, R⁶, and R^{6*} is independently selected, preferably from hydrogen, C₁₋₁₂-alkyl, C₂₋₁₂-alkenyl, C₂₋₁₂-alkynyl, hydroxy, C₁₋₁₂-alkoxy, C₂₋₁₂-alkenyloxy, mercapto, C₁-C₁₂ alk(en)ylthio, carboxy, C₁₋₁₂-alkoxycarbonyl, C₁₋₁₂-alkylcarbonyl, formyl, aryl, aralkyl, aryloxy-carbonyl, aryloxy, arylthio, aralkoxy, arylcarbonyl, heterocyclyl, heterocyclyl-alkyl, heterocycliloxy-carbonyl, heterocyclyl-oxy, heterocyclyl-carbonyl, amino, mono- and di(C₁₋₆-alkyl)amino, carbamoyl, mono- or di(C₁₋₆-alkyl)-amino-carbonyl, amino-C₁₋₆-alkyl-aminocarbonyl, mono- or di(C₁₋₆-alkyl)amino-C₁₋₆-alkyl-aminocarbonyl, C₁₋₆-alkyl-carbonylamino, carbamido, C₁₋₆-alkanoyloxy, sulphonyloxy, C₁₋₆-alkylsulphonyloxy, nitro, azido, sulphonyl, C₁₋₆-alkylthio, halogen;
30
35

INTERNATIONAL SEARCH REPORT

International Application No
PCT/DK2004/000372

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C07H19/06 C07H19/10 C07H19/16 C07H19/20 C07H21/00
C12N15/11 A61K31/7064 A61K31/7076 A61K31/712 A61P31/12
A61P35/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C07H C12N A61K A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	X. WU, S. PITSCH: "Synthesis and pairing properties of oligonucleotide analogues containing a metal-binding site attached to beta-D-allofuranosyl cytosine" NUCLEIC ACIDS RESEARCH, vol. 26, no. 19, 1998, pages 4315-4323, XP002293167 introduction compounds 14, 15 tables 1, 2	1-4,6, 13,14, 16-20, 23,24
X	WO 95/18817 A (UNION PHARMA SCIENT APPL ; BRU.MAGNIEZ NICOLE (FR); GUENGOER TIMUR (FR) 13 July 1995 (1995-07-13) page 1 page 6 examples 6, 53, 69	1,23,24

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☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

- *A* document defining the general state of the art which is not considered to be of particular relevance
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- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

- *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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- *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- *A* document member of the same patent family

Date of the actual completion of the international search

27 August 2004

Date of mailing of the international search report

10/09/2004

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/DK2004/000372

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 00/66604 A (EXIQON AS ; WENGEL JESPER (DK)) 9 November 2000 (2000-11-09) the whole document	1,13,23
P,X	M. RAUNKJAER ET AL.: "N-Methylpiperazinocarbonyl-2',3'-BcNA and 4'-C-(N-methylpiperazino)methyl-DNA: introduction of basic functionalities facing the major groove and the minor groove of a DNA:DNA duplex" CHEM. COMM.; 2003, pages 1604-1605, XP002293168 the whole document	1-27

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/DK2004/000372

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			US 2003087230 A1	08-05-2003